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THE HISTOLOGY OF ADAMANTINOMA.

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THE adamantinoma is one of the less common tumours of the jaw. Its long natural history and marked tendency to recur after inadequate surgery or radiotherapy make an evaluation of methods of treatment difficult. It is important, therefore, that as many detailed cases as possible should be recorded. In this paper the clinical and pathological features of 20 cases are presented and the histogenesis of the tumour is discussed.

CLINICAL FINDINGS.

These tumours occur most often in early adult life, though cases have been reported in children, and in one case in this series the age of onset was 78. The tumour occurs in the lower jaw much more frequently than in the upper (17 cases and 3 cases respectively in this series), is usually a central lesion and gradually enlarges, in the majority of cases without pain. In the course of time the bone becomes absorbed, and if a cystic loculus is opened infection and discharge follow.

Local recurrence is common following inadequate treatment, but metastases are very rare. A case of pulmonary metastases has been reported by Waterworth and Pullar (1948), who review previous reports.

The natural history of the disease is long; it is not always easy therefore to assess the efficacy of various methods of treatment. Most authorities consider radical excision of the tumour to be the treatment of choice. The tumour, if small, is removed with a surrounding margin of healthy tissue, though extensive lesions require resection of the jaw (Simmons, 1928; Stones, 1948; Thoma, 1948). Radiotherapy gives unsatisfactory results.

Table I summarizes the clinical findings in our series.

HISTOLOGY.

The adamantinoma is an epithelial tumour in which the cellular configuration may take a variety of forms, according to the degree of differentiation of the component cells. Thus the least evolved type of tumour consists of strands of more or less undifferentiated epithelial cells growing in a fibrous stroma, while a tumour which has undergone a greater degree of differentiation consists of follicles

or nests of cells approximating in appearance to the developing enamel organ (Fig. 1). The epithelial follicles in such cases consist of an outer layer of columnar cells resembling the similarly situated cells of the enamel organ, while towards the centre the cells become stellate in appearance and cyst formation may occur. On the other hand, the trend of differentiation may be towards a more highly developed type of squamous epithelium, in which case the tumour consists of sheets of squamous cells showing a tendency to cornification, or a basal cell type of growth may be produced.

These tumours may thus present a variety of appearances, from case to case, or in different areas of the same growth, and while attempts at classification on a histological basis are helpful in so far as they indicate the varying degrees of differentiation which can be observed, they are seldom entirely applicable to any given specimen. Thoma (1950) has proposed one such classification, as follows, though he notes that pure types are but infrequently seen:

(1) *Primitive type*, composed of strands of epithelial cells showing little tendency to differentiation, growing in a loose fibrous stroma. When the stroma takes on neoplastic characteristics the tumour is classified as ameloblastofibroma. (2) *Plexiform type*, consisting of strands of cells more highly differentiated than those of the primitive type. (3) *Stellate type*, in which the cells have reached the stellate shape seen in the enamel organ. (4) *Follicular type*, consisting of follicles composed of cells similar to those seen in a rather more advanced stage of development of the tooth germ. At the periphery of the follicles the cells are cylindrical, resembling ameloblasts. (5) *Acanthoma type*. In this type differentiation into squamous cells occurs. (6) *Adeno-ameloblastoma*. The epithelium is arranged in a glandular pattern. (7) *Haemangio-ameloblastoma*. A very rare type of tumour in which haemangiomatous characters are present. (8) *Melano-ameloblastoma*. Also a very rare tumour, in which the epithelial cells contain pigment.

In our series 4 cases were of the primitive type, whilst the remainder were largely follicular or showed mixed appearances. Fig. 2 (Case 5) shows an example of the primitive type of tumour, cords of epithelial cells growing in a fibrous stroma. At some points there is evidence of commencing differentiation. Similar appearances were seen in Cases 6 and 11. In Case 19 the tumour showed a very cellular stroma (Fig. 3) resembling the mesenchyme of the dental pulp, and can be considered as an adamantinofibroma.

In Case 3 (Fig. 4) the growth is composed of solid masses of cells, though in some areas there is differentiation of the epithelium towards a more columnar type. Case 16 (Fig. 5) shows differentiation of a solid type of growth towards squamous epithelium, with areas of cornification.

The more highly differentiated type of tumour is seen in Fig. 6 (Case 5), in which the growth is composed of follicles or nests of epithelial cells with outer columnar layer and inner stellate area.

Microcyst formation, a common occurrence in adamantinoma, was present in 14 cases. Typically, these cysts occur in the area of stellate cells towards the centres of the follicles, but they also form in the stroma. The occurrence of stromal cysts is a point to which very little previous consideration appears to have been given. Siegmund (1929) noted their occurrence in 2 of his cases, but apart from this instance, and a passing reference by Kronfeld (1930) to the same cases, the literature is silent on the matter. Fig. 7 (Case 14) shows microcysts forming in an epithelial mass, and numerous microcysts are also present in the

tumour shown in Fig. 8 (Case 1). In the latter case, however, it will be seen that though there is some breakdown within the follicles, the majority of the cysts have formed on the side of the columnar cell layer opposite to that on which stellate cells are situated. Fig. 9 shows three such cysts in higher magnification. A further example of this type of cyst formation can be seen from another field in Case 14 (Fig. 10, 11). In these cases can be seen evidence of the degenerative changes which precede cyst formation. Thus many of the stromal enclaves show a mucinous type of degeneration, the connective tissue becoming very loose and staining pale blue with haematoxylin. In other precystic areas of stroma foam cells are present. Of these 14 cases showing cyst formation, this change occurred in the epithelium only in 6 cases (Cases 2, 4, 7, 9, 17, 18), in both epithelium and stroma in 7 cases (Cases 1, 3, 8, 10, 14, 15, 20), and in the stroma alone in one case (Case 11). Even in the 6 cases showing epithelial cysts only, evidence could be found in 3 cases of incipient degenerative changes in the stroma.

The cause of cyst formation is a matter of conjecture. It is believed to be the result of progressive degeneration in originally solid growths. The solid tumour closely resembles the developing tooth up to the point at which differentiation of adjacent connective-tissue cells into odontoblasts, and subsequently amelogenesis, would occur in the case of the tooth, but at this stage the tumour cannot develop further. Degeneration therefore takes place (Kronfeld, 1930 ; Robinson, 1937*a*). The ensuing accumulation of fluid of relatively high osmotic tension is probably a factor in the progressive enlargement of the microcysts, a mechanism investigated by Toller (1948) in the case of simple dental cysts, and quite possibly a factor also in the case of cyst formation in these tumours.

With regard to cyst formation in the stroma, it appears that again a process of degeneration is at work, and all stages of breakdown can be seen from a slight loosening of the stroma to mucinous-like degeneration and the formation of completely clear cystic spaces. Though similar degeneration leading to cyst formation may also be observed in basal cell carcinomata the phenomenon is not peculiar to tumour growth as such, since the earlier stages of a similar occurrence can be observed not infrequently in the connective tissue underlying the proliferating epithelium of dental cysts. In fact, it is this stromal degeneration in simple cysts which occasioned Churchill (1934) to emphasize that such areas should not be mistaken for degeneration in the stellate reticulum of adamantinomatous follicles.

HISTOGENESIS.

The teeth develop as compound structures, the enamel being derived from the oral ectoderm, the dentine, cementum and pulp from mesoderm.

At an early stage in embryonic development an extension of the oral epithelium, the dental lamina, grows into the mesenchyme of the jaw. Ten bud-like thickenings appear at intervals on the lamina, the primordia of the enamel organs for the deciduous teeth, and the cells of the mesenchyme under the buds become condensed to form the primordia of the dentine papillae. Further growth of the lamina leads to the production of similar buds for the permanent teeth, one on the lingual side of each deciduous germ, and those for the permanent molars. The tooth buds thus formed continue to proliferate, producing domed structures with indented deep surfaces surmounting the papillae. The epithelium becomes differentiated into a peripheral layer and a central area, the former constituting

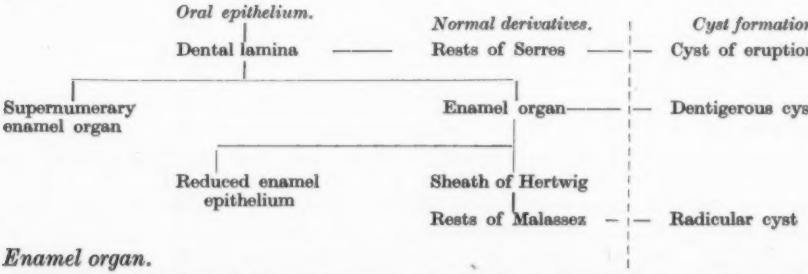
the enamel epithelium and the latter the stellate reticulum. The enamel epithelium consists of inner and outer divisions. The inner enamel epithelium is that which lines the concavity of the enamel organ, a single layer of tall columnar cells which will produce enamel, while the outer enamel epithelium consists of cuboidal cells covering the convexity of the organ. The central area of the enamel organ, the cells enclosed by the continuous layers of internal and external enamel epithelium, is the stellate reticulum. The cells here have become separated by intercellular fluid and resemble a network of reticular connective tissue. Between the stellate reticulum and the inner enamel epithelium are several layers of flattened cells, the stratum intermedium.

After the germs are formed in the manner described the dental lamina disintegrates, leaving only isolated groups or nests of epithelial cells between the oral epithelium and the enamel organ, the epithelial rests of Serres.

Enamel is formed by the cells of the inner enamel epithelium, though this cannot take place till the cells of the underlying dentine papilla have differentiated to form odontoblasts and produced dentine. Huggins, McCarroll and Dahlberg (1934) have shown by transplantation experiments that enamel is laid down only on dentine, while Glasstone (1935) demonstrated in tissue culture that odontoblasts can differentiate from mesenchymal tissue only in the presence of enamel epithelium. However, Thoma and Goldman (1946) state that dentine formation is independent of ameloblasts. During the course of enamel formation the stellate reticulum undergoes reduction and finally disappears, so that when the enamel is completely developed the ameloblasts and the cells of the outer enamel epithelium have come into contact, to form the reduced enamel epithelium.

Development of the roots takes place under the influence of Hertwig's epithelial root sheath. This structure is formed by the reflection of the two layers of enamel epithelium at the " mouth " of the bell-shaped enamel organ, and the cells of the inner layer induce the differentiation of connective-tissue cells into odontoblasts. When this has occurred and the first layer of dentine has been deposited, the epithelial sheath breaks up. Its remnants persist as the epithelial rests of Malassez.

Thus the oral ectoderm gives rise to a number of structures in the course of development, from any of which, theoretically, an epithelial tumour can originate. The oral epithelium and its derivatives can be diagrammatically represented, thus :



Enamel organ.

The striking resemblance of many adamantinomata to the enamel organ led to the earliest of the theories of origin of these tumours. Broca (1868) considered them to be closely related to the enamel organ, and many writers since have held

this opinion. On the other hand, Bland-Sutton (1922) thought that the enamel organ could not be the origin of these growths, since the majority of patients were well into adult life when their tumours first appeared. However, Robinson (1937*b*) in his survey of 379 cases found that the tumour was first noticed between the tenth and thirty-fifth years in 70 per cent of cases. Considered together with the fact that these tumours are very slow growing and have a long natural history, it is evident that the majority of growths originate at quite an early age. Robinson (1937*b*) also found that the tumour occurred most frequently in the premolar and molar regions, where supernumerary tooth germs most often occur, and that many cases were associated with a missing tooth. Kegel (1932) and Geschickter (1935) also support the view that the tumour arises from the enamel organ. Byers and Sarnat (1945) believe that the tumour originates from that structure during the first few years of life, though active growth may not become manifest till later.

If these tumours, or at least the majority of them, originated directly from the enamel organ, it might be expected that on occasion enamel would be formed. This is never the case, though Thoma (1950) considers that the homogeneous zone sometimes to be seen between the basal membrane of the columnar cells in the follicular type of adamantinoma and the stroma, which appears yellow with Van Gieson's stain, must be considered as abortive enamel formation, though it is possible that precystic changes in the stroma could account for such appearances. Thus to ascribe the origin of the tumour to the enamel organ directly implies that the tumour must originate at an early stage, prior to amelogenesis. It must then remain dormant, in some cases for a considerable time.

The occurrence of tumours similar in structure to the adamantinoma in the tibia and in the pituitary must also be considered. In the case of the former the resemblance is probably coincidental (Willis, 1948), though as the pituitary is connected embryologically with the stomatodoeum it is not surprising that tumours derived from it can resemble epithelial tumours of the jaws. But no enamel organs occur in the hypophysis.

A tumour similar in some respects to the adamantinoma, which does produce enamel, is the adamantino-odontoma. This is a rare tumour, though it is the type of growth which might be expected to occur more frequently were differentiated enamel organ epithelium a common source of tumour origin.

Oral mucosa.

It has been held that the tumour arises from the oral mucosa. Siegmund and Weber (1926) found numbers of cases in which there was a connection between the oral mucosa and the tumour epithelium, but they were of the opinion that the tumour might equally well have grown up to and established connection with the surface epithelium as have originated from it. Cases of this type have also been reported by Gullifer (1936), Robinson (1937*a*), Fish (1948), Bernstein (1949), Champion, Moule and Wilkinson (1951). Some tumours show areas closely resembling basal cell carcinoma; Sprawson and Keizer (1933) and Sprawson (1937) believe that the adamantinoma is in fact to be considered as of such nature.

In our series there are 5 cases showing connection with the surface epithelium. An example is Case 1 (Fig. 12), the tumour cells being connected with the surface epithelium over a wide area.

The long duration of the adamantinoma, in most cases as a completely central

tumour without involvement of the oral epithelium, is, however, evidence against an origin from the oral mucosa.

These tumours resemble rodent ulcers in several respects. Both are related to derivatives of the covering ectoderm, the one to teeth and the other to hair follicles, though discussion continues as to the stage of development of the appendage at which they arise. Do they come from the fully developed appendage, from surface epithelium which is destined to form such appendages, or from epithelial rests ? The growths also resemble each other in their natural history, growing slowly and rarely if ever metastasizing ; their histological appearance, too, is comparable, and intercellular accumulation of fluid in a rodent ulcer may give it a striking resemblance to the more solid type of adamantinoma.

Cysts of dental origin.

A number of cases in which adamantinoma has been considered to have originated in the epithelial lining of a dentigerous cyst have been reported (Schroff, 1931 ; Cahn, 1933 ; Carpenter and Thoma, 1933 ; Thoma and Proctor, 1937). These cases lack the exact proof of histological examination before and after development of the tumour, so that the possibility of the cysts having been of adamantinomatous nature from the outset cannot be excluded. Nevertheless, the evidence given does justify as a presumption the origin of tumours, in some cases, from cyst linings.

Epithelial rests.

Malassez first demonstrated the presence of epithelial rests about the roots of the teeth and postulated these as the origin of adamantinomata. Kegel (1932) did not think that the rests of Malassez could give rise to these tumours since, as was then thought, Hertwig's sheath, from which the rests are derived, was composed of outer enamel epithelium only. It is now considered, however, that both layers of enamel epithelium participate in its structure (Orban, 1949).

Epithelial rests appear to be the most likely site of origin for the majority of adamantinomata. They are composed of relatively undifferentiated cells which can give rise to any of the appearances found in these tumours, and moreover will produce a central growth. It is not necessary to postulate tumour formation from these rests in early life, with a subsequent interval of dormancy to account for those cases in which the tumour first appears at a comparatively late age. Being present from the formative phase of the dental tissues onwards they can proliferate at any time, and this seems a more satisfactory hypothesis than the dormancy theory.

The rests of Serre, those left by the dental lamina, are situated quite superficially. They are therefore less likely to be the origin of adamantinomata than the rests of Malassez, which are situated more deeply, within bone.

Nomenclature.

Most discussions on the subject of adamantinoma refer to the inappropriate terminology in current use ; it is admittedly difficult to find an acceptable nomenclature for these tumours. The old terms " multilocular cyst " and " epithelial odontome " have rightly been abandoned, while " adamantinoma " and " ameloblastoma " are misnomers, since neither is enamel formed nor are the tumour

cells ameloblasts. Willis (1948) suggests " carcinoma of the tooth germ residues " as an appropriate title ; certainly it is free from the objections mentioned above. We have, however, adhered to the more familiar though inaccurate eponym for the sake of brevity.

Case Histories.

Case 1 : *S. D—, female, b. 1854.—1932 :* The patient first reported with a small polyp inside the left cheek. This was removed after X-ray of the jaw had shown nothing abnormal. *1933 :* Pain was now experienced in the region of the left temporo-mandibular joint and a hard, smooth swelling was found on the outer side of the left superior alveolar margin. The left maxilla was removed and radium treatment commenced. *1934 :* Condition satisfactory. No recurrence. *1936 :* Died. *Histology.*—This is a tumour of the stellate type, with plexiform areas. Numerous microcysts are present, both in the epithelium and in the stroma. Some of the latter contain foam cells. The tumour epithelium is connected with that of the surface over quite a large area.

Case 2 : *W. H—, male, b. 1866.—1880 :* " Jaw scraped." Repeated 4 times in 8 or 9 years. *1928 :* Swelling of right lower jaw of 7 weeks' duration. *1929 :* Much increased in size. Cyst opened. Radon collar, 61·6 mc. in 14 tubes for 6 days. *1936 :* Condition very satisfactory. *Histology.*—The tumour is of the typical follicular variety, showing cystic degeneration within the follicles.

Case 3 : *A. G—, female, b. 1880.—1937 :* Tumour of the left mandible 4½ in. in diameter, of many years' duration. Left mandible removed, from symphysis to upper part of ascending ramus. *1951 :* Very well. *Histology.*—Microscopic examination shows large sheets of polygonal cells in a scanty fibrous stroma. At places the continuous sheets break up to some extent, to form a plexiform arrangement. Here the cells tend to take on a stellate appearance towards the centres of the cords and become elongated and columnar at the periphery. There is some cystic degeneration in the stellate areas and also in the stroma.

Case 4 : *M. H—, female, b. 1893.—1910 :* Dentigerous cyst of one year's duration removed. *1924 :* Recurrence of swelling. *1928 :* Swelling had gradually increased in size till there was now a hard tumour of the right mandible. This was excised and the cavity of a multilocular cyst exposed. The contents were removed. *1931 :* The swelling has gradually recurred. There has always been some pain. There is now a large, hard swelling on the right side of the face adherent to the mandible. Posteriorly it contains a cavity about 0·5 cm. in diameter. X-ray shows expansion of the bone and destruction within the swelling. The right half of the mandible was resected. *1951 :* Condition satisfactory. No recurrence. *Histology.*—This tumour is of the follicular type, consisting of fairly regular nests of stellate cells bounded by columnar epithelium. Cyst formation in the central cells is prominent. One area shows a connection between tumour and surface epithelium.

Case 5 : *C. D—, male, b. 1885.—1905 :* Blow in face followed by painful swelling of left cheek, which was opened intrabucally. *1907, 1912, 1914 :* Three intrabuccal operations for recurrence of swelling. *1914 :* Recurrence. Opened from exterior. *1916 :* Recurrence. *September, 1925 :* There has been progressive swelling of left superior maxilla since 1916. The left side of the hard palate is also swollen and bulges down on to the tongue. All teeth have been removed. Antrum incised. 2 × 50 mg. radium tubes inserted. 2 × 50 mg. radium tubes forced up through hard palate. Tubes left in 18 hours. *October, 1925 :* No improvement. *December, 1925 :* Left side of face swollen over superior maxilla. Severe pain. On left side of hard palate is an irregular swelling extending from mid-line to, and including alveolar margin. *January, 1926 :* Alveolar part of superior maxilla and the palatal tumour excised. Radium 75 mg.

for 12 hours. *May, 1926:* Much improved. *1931:* Condition satisfactory. No recurrence. *1937:* Condition satisfactory. *October, 1939:* Swelling of right side of face. The right antrum is expanded and there is a projection into the right labio-alveolar sulcus. There is evidence of growth at the posterior end of the left maxilla. The right side of the palate is pushed down and irregular in outline. Twenty-eight treatments of deep X-rays given over 38 days. Total tumour dose 6003 r. *June, 1940:* Sequestrum in temporo-mandibular region and abscess in temporal fossa. Pain very severe. Sequestrectomy and abscess evacuated. *December, 1940:* Died of broncho-pneumonia, with osteomyelitis and tumour of jaw. *Histology.*—The tumour is composed of strands of comparatively undifferentiated cells in a cellular fibrous matrix.

CASE 6: *C. S—, male, b. 1887.—1923:* A large multilocular cyst was found in the left mandible. The cyst was opened and a wisdom tooth removed. *1924:* A further operation revealed a cavity the size of a walnut in the horizontal ramus. The cyst contained a mass of tissue resembling a villous papilloma. The cyst was enucleated and packed. Untraced since. *Histology.*—The tumour consists of strands and masses of comparatively undifferentiated cells in a fibrous stroma.

CASE 7: *H. B—, male, b. 1900.—May, 1937:* Admitted to hospital with large swelling in $\overline{43}$/ region, of 2 months' duration. Discharge of sero-pus on pressure. No pain. *June, 1937:* Cyst in $\overline{543}$/ region of right mandible removed. *May, 1951:* Patient has been perfectly well since the operation. *Histology.*—The tumour is composed of large masses of epithelium arranged, in places, in follicles. The epithelium is of the stellate type, with the outer layers of the masses and follicles consisting of cuboidal or columnar cells. Microcysts are present, though in comparatively small number, in the epithelium. At one point there is a connection between surface and tumour epithelium.

CASE 8: *M. S—, male, b. 1867.—1927:* Tumour of right mandible of 6 years' duration. Radium treatment commenced, but not completed. *1934:* Now has a large tumour on the right side of the mandible. X-ray examination shows the mandible to be irregularly expanded by multiple cyst formation. Surface application of radium, 13,440 mgh. The following month a surface application of 22,038 mgh. was given. *1935:* Tumour still present though smaller. *1937:* Tumour large. Radium treatment commenced, but terminated by patient before completion. *1939:* Committed suicide. *Histology.*—This is a growth of the follicular type, the cellular masses consisting of stellate cells bounded by columnar epithelium. Numerous cysts are present, mainly

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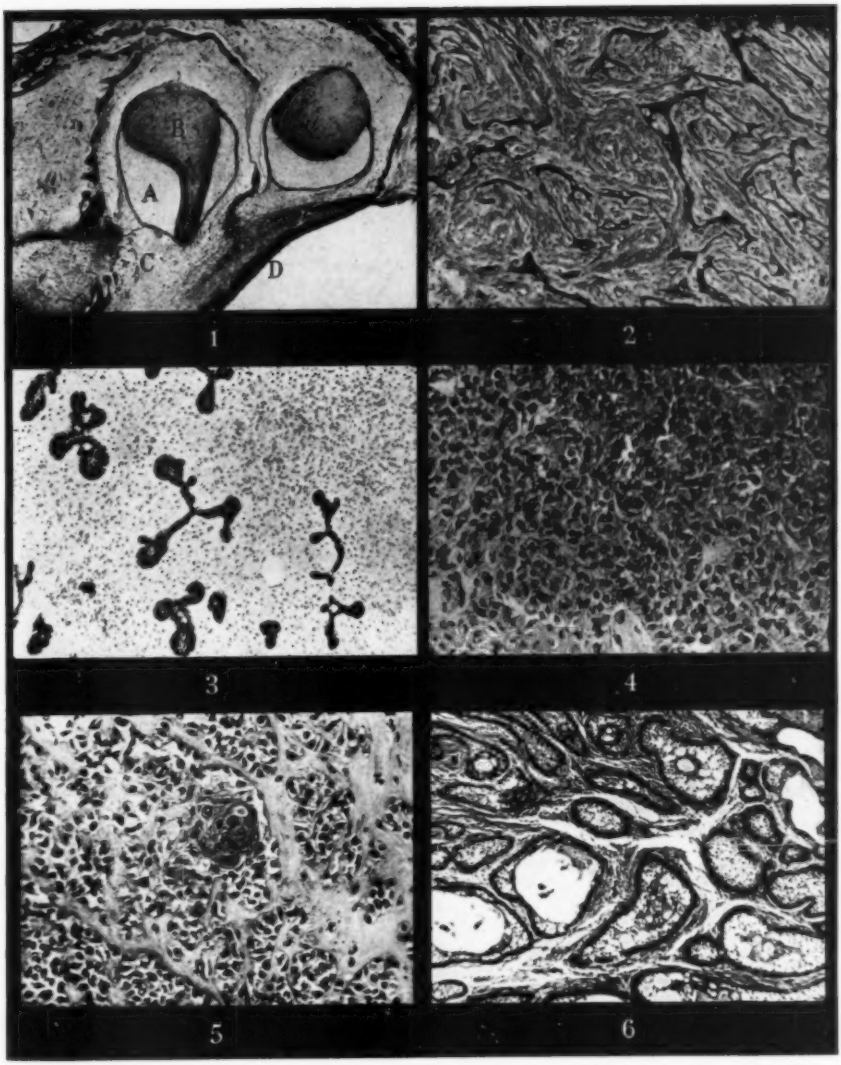
FIG. 1.—Developing tooth germs.
A. Stellate reticulum.
B. Dentine papilla.
C. Remains of dental lamina.
D. Oral epithelium.
The germ on the left is the older, showing deposition of enamel and dentine. × 9.
FIG. 2.—A primitive type of tumour. The epithelial component consists of strands of poorly differentiated cells. × 60.
FIG. 3.—Adamantinofibroma. The stroma is very cellular. × 60.
FIG. 4.—A cellular type of tumour. × 170.
FIG. 5.—Another cellular type of tumour showing an area of keratinization. × 175.
FIG. 6.—The typical follicular variety. The follicles are composed of a layer of columnar epithelium surrounding cells of stellate appearance. × 60.
FIG. 7.—Microcyst formation. The cyst formation is due to breakdown of the stellate cells × 75.
FIG. 8.—Microcyst formation. Numerous microcysts are present, mainly in the stroma. × 50.
FIG. 9.—A field from Fig. 8. The microcysts occur on the side of the columnar cell layer opposite to that on which the stellate cells are placed. Small cysts are also present in the stellate area. × 175.
FIG. 10.—Another example of microcyst formation in the stroma. × 25.
FIG. 11.—A field from Fig. 10. × 65.
FIG. 12.—Connection between tumour and surface epithelium. × 175.



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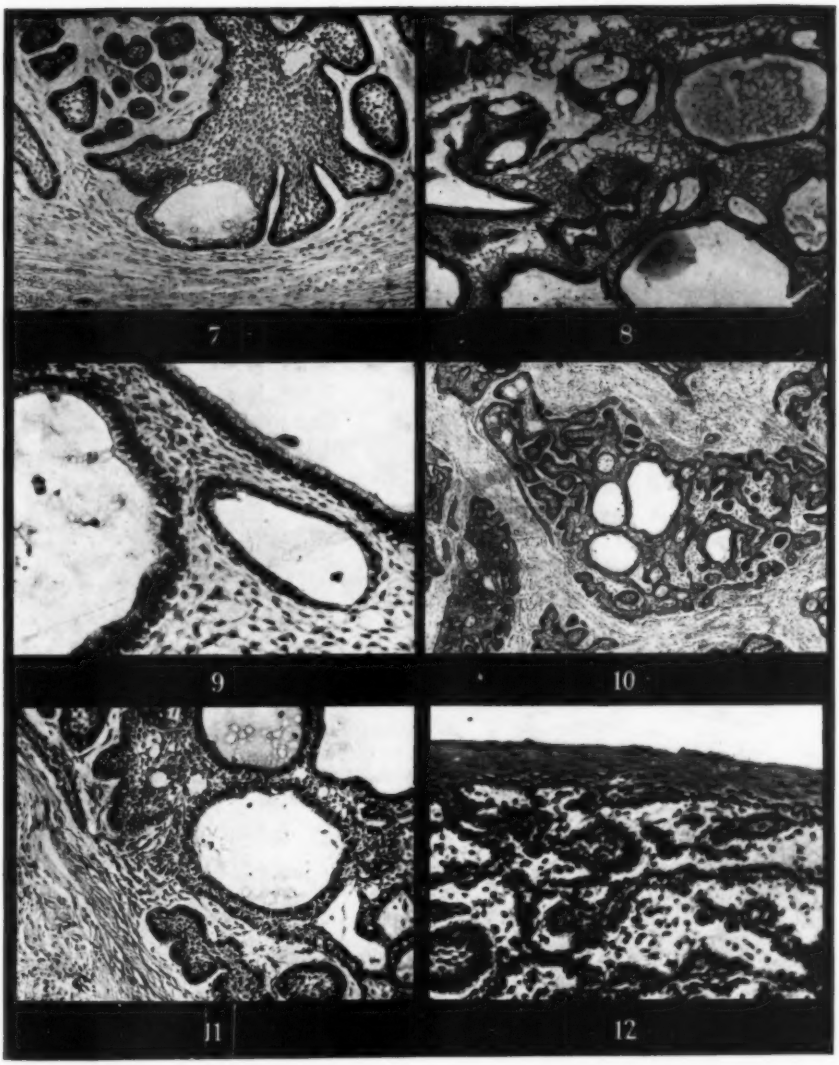

7 8

9 10

11 12

in the stroma. In some areas cyst formation is not quite complete, and the preceding mucinoid degeneration of the stroma is well seen.

CASE 9: *D. W—, female, b. 1905.—1935:* Cyst removed from /4–8 region. No histological examination made. *1940:* The patient complains of dull pain in left mandible. There is a large, fluctuating swelling in /6—→ region. X-ray showed loculated cyst. The cysts were "saucerized" and packed. A plug was later inserted. *1951:* Very well. No recurrence. *Histology.*—Microscopic examination shows follicles of stellate cells bounded by a layer of columnar epithelium in an abundant, quite dense, stroma. The follicles show central cyst formation.

CASE 10: *B. M—, female, b. 1892.—1926:* Noticed a lump in the right upper jaw which she thought to be due to ill-fitting dentures. This was treated with "caustic" and disappeared, but recurred within 4 months. It was again similarly treated. *1928:* On admission to hospital an oval area of enlargement of the right maxilla was noted, situated between the alveolar margin of the maxilla and the inner surface of the cheek, the long axis directed antero-posteriorly for about an inch. The area of enlargement and the maxillary antrum were opened up and curetted. A 50-mg. radium tube was placed in the cavity for 24 hours. Untraced since. *Histology.*—The tumour is of the follicular type in most areas, though in some parts it takes the form of fairly large continuous sheets of epithelium. Cystic degeneration is present in the epithelium and in the stroma.

CASE 11: *D. D—, female, b. 1905.—1947:* Occasionally felt some stiffness in the left side of the jaw. *1948:* A lump in the jaw. *1949:* The lump has now begun to discharge. On examination there is a hard mass along the inferior border of the mandible in the /3–6 region, extending upwards to the external surface of the bone to the superior border. /4567 had been removed. Left half of mandible resected. *1951:* No recurrence. General condition satisfactory. *Histology.*—The tumour is composed of strands of epithelial cells in a connective-tissue stroma. The cells are for the most part polyhedral or spheroidal, but in areas where the epithelial strands are thin they tend to be flattened. The stroma is quite loose and shows mucoid degeneration, with the formation of microcysts.

CASE 12: *E. W—, female, b. 1899.—1928:* Operated on for "abscess of jaw." *1937:* Painful swelling appeared at site of previous operation. X-ray examination showed a multilocular cyst in the 3–/–6 region. The cyst was opened. *1940:* Swelling recurred and cyst again opened and contents removed. *1943:* No recurrence. *1951:* Recurrence, but very small. *Histology.*—The tumour consists of islets of epithelial cells in a plentiful, fairly dense, fibrous stroma. The epithelial islets consist of an outer layer of cubical cells enclosing a central stellate area.

CASE 13: *F. B—, female, b. 1887.—1929:* The patient complained of a lump on the right side of the jaw of 10 years' duration, which had been operated on 3 times, 10, 6 and 3 years previously. It had increased in size, painlessly, but rather rapidly since the last operation. On examination there was a large swelling on the right side of the mandible, showing the typical X-ray appearances of adamantinoma. The right side of the mandible was removed. *1951:* Condition satisfactory. *Histology.*—Microscopic examination shows typical epithelial clumps lined by columnar cells and containing stellate cells in the central areas. Cyst formation is well marked. The stroma is quite plentiful and fairly dense, though around some of the epithelial islands it tends to be looser and shows evidence of degeneration.

CASE 14: *A. W—, male, b. 1881.—1937:* Three years' history of swelling of the right lower jaw after extraction of 3 teeth. This was considered to be due to a dental cyst, and the jaw was scraped on two occasions. After the first operation the swelling disappeared. On its recurrence a second scraping was performed, but the swelling persisted and increased in size, especially during the last year. There was never any

pain. Examination showed a large swelling on the lingual and buccal aspects of the premolar and molar regions. X-ray showed expansion of the jaw by a loculated, cystic area. The mandible was resected. *1950 :* Died from other causes. No recurrence. *Histology.*—The tumour is composed of typical epithelial nests with reticular centres, showing in some areas cystic degeneration. The majority of the cysts seen in this tumour, however, occur in the stroma.

Case 15 : *E. N—, male, b. 1904.—1945 :* Cyst in right lower jaw, of 6 months' duration. Two teeth and cyst removed. *1947 :* There has been residual swelling in the jaw since removal of the cyst. This has now started to enlarge. X-ray examination shows entire ramus occupied by cyst, up to the coronoid process. Right half of mandible resected. *1950 :* No recurrence. General condition satisfactory. *Histology.*—The tumour shows typical epithelial follicles in a fibrous stroma. Many of these are undergoing central cyst formation, and there are also some cysts in the stroma.

Case 16 : *D. H—, male, b. 1883.—1937 :* Admitted to hospital for a fracture of the elbow-joint, when a large swelling of the right mandible was noticed. This appeared to have started some 5 years previously, and had been gradually increasing in size. X-ray examination showed the typical appearances of adamantinoma, and local excision of the cyst was undertaken. The patient died a few years later from other causes. *Histology.*—The tumour is composed of masses of oval or pyriform cells showing a tendency towards an alveolar type of arrangement. Areas of keratinization are present. The stroma is dense and shows comparatively few nuclei ; it is hyaline at some points. Necrosis and haemorrhage occur in a number of areas, and involve both epithelium and connective tissue equally.

Case 17 : *M. K—, female, b. 1905.—1923 :* Tooth removed from left lower jaw ; the socket did not heal properly. *1938 :* Dental cyst removed from same region. *1946 :* Enlargement of the left mandible, painless and not tender. X-ray examination showed a large cyst extending well up into the ramus. *1948 :* Left half of mandible removed. *Histology.*—The tumour consists of epithelial follicles bounded by columnar epithelium. The follicles contain stellate cells and show microcyst formation.

Case 18 : *H. B—, male, b. 1876.—1927 :* Noticed a swelling in the jaw. This was operated on. *1931 :* Growth recurred. Operation. *1937 :* Recurrence. On examination there was a large, firm mass, 3·5 cm. in diameter, below the angle of the mandible on the left side. Behind this was a smaller mass 1 cm. in diameter. Both were fixed to the deep tissues, but not to the skin. Deep X-rays given over 4 fields in 15 treatments with a total of 5200 r. Second course given 6 months later over 3 fields in 21 treatments. Total dose 6600 r. *1939 :* Mass still present, but patient very well. *1940 :* Condition satisfactory. Mass still present but smaller, and apparently composed of sclerotic bone only (by X-ray). *1946 :* Very well. No recurrence. *Histology.*—The tumour consists of typical epithelial follicles in a fibrous stroma. Microcyst formation is present in the follicles.

Case 19 : *E. S—, female, b. 1929.—1946 :* Swelling of left side of mandible of 6 weeks' duration. No pain. On examination there is an ovoid area of expansion in the $\overline{|456}$ region. Segment of mandible excised. *1951 :* Very well. No recurrence. *Histology.*—The tumour is composed of islets of cubical epithelial cells in a very cellular stroma.

Case 20 : *K. C—, male, b. 1927.—1951 :* Noticed a small swelling in the lower jaw 3 years ago. This has become progressively larger and recently it has been painful. On examination there is a swelling 2½ in. in diameter extending from the body of the mandible along the ramus. X-ray showed a loculated cyst, and at operation the cyst was opened and the lining removed. *Histology.*—The tumour consists of areas of stellate cells bounded by a single layer of columnar epithelium. There is cyst formation both in the epithelium and in the stroma.

TABLE I.

Case.	Sex.	Site.	Age at onset.	Age at main treatment.	Treatment.	Result.
1	F.	Maxilla	78	79	Resection of maxilla and radium	No recurrence after 1 yr.
2	M.	Mandible	First jaw symptoms at 8 yrs. Tumour appeared 40 yrs. later	63	Radon	,, ,, 7 yrs.
3	F	,,	?	57	Resection of mandible	,, ,, 10 yrs.
4	F.	,,	Dentigerous cyst at 17 yrs. Tumour appeared at 31 yrs.	38	Ditto	,, ,, 20 yrs.
5	M.	Maxilla	20	40 54	Radium X-rays	No further information.
6	M.	Mandible	36	37	Enucleation of cyst	,, ,,
7	M.	,,	37	37	Ditto	No recurrence after 14 yrs.
8	M.	,,	54	60 67 70	Radium ,, ,,	Tumour still present.
9	F.	,,	30	35	Saucerization of cyst	No recurrence after 11 yrs.
10	F.	Maxilla	34	36	Radium	No further information.
11	F.	Mandible	42	44	Resection of mandible	No recurrence after 2 yrs.
12	F.	,,	29	29 38 41	Enucleation of cyst	Recurrence after 11 yrs.
13	F.	,,	32	42	Resection of mandible	No recurrence after 22 yrs.
14	M.	,,	53	56	Ditto	,, ,, 13 yrs.
15	M.	,,	41	43	,,	,, ,, 3 yrs.
16	M.	,,	49	54	Excision of cyst	No recurrence after some years.
17	F.	,,	?	41	Resection of mandible	No further information.
18	M.	,,	51	61	X-ray	No recurrence after 9 yrs.
19	F.	,,	17	17	Resection of mandible	,, ,, 5 yrs.
20	M.	,,	24	24	Removal of cyst lining	To be followed by excision of mandible.

SUMMARY.

1. Twenty cases of adamantinoma are described.

2. The histology of the tumour is discussed and the stromal origin of some of the cysts is emphasized.

3. The histogenesis of these tumours is considered. Reasons are given for believing that they originate in the rests of Malassez in most cases.

We are indebted to those surgeons of the Royal Dental Hospital and the Middlesex Hospital whose cases we have studied, and particularly to Mr. D. Greer Walker. Part of the expenses of this study were defrayed by the British Empire Cancer Campaign.

REFERENCES.

BERNSTEIN, E.—(1949) *Oral. Surg., Oral Med., Oral Path.*, **2**, 726.

BLAND-SUTTON, J.—(1922) 'Tumours, Innocent and Malignant.' London (Cassell & Co., Ltd.).

BROCA, P.—(1868) *Gaz. hebd. d. sc. méd. de Bordeaux*, **25**, 70.

BYERS, L. T., AND SARNAT, B. G.—(1945) *Surg. Gynec. Obstet.*, **81**, 575.

CAHN, L. R.—(1933) *Dent. Cosmos*, **75**, 889.

CARPENTER, L. S., AND THOMA, K. H.—(1933) *Dent. Items*, **55**, 716.

CHAMPION, A. H. R., MOULE, A. W., AND WILKINSON, F. C.—(1951) *Brit. dent. J.*, **90**, 6.

CHURCHILL, H. R.—(1934) *Dent. Cosmos*, **76**, 1173.

FISH, E. W.—(1948) 'Surgical Pathology of the Mouth.' London (Pitman).

GESCHICKTER, C. F.—(1935) *Amer. J. Cancer*, **26**, 90.

GLASSTONE, S.—(1935) *J. Anat.*, **70**, 260.

GULLIFER, W. H.—(1936) *Dent. Cosmos*, **78**, 1256.

HUGGINS, C. B., MCCARROLL, H. R., AND DAHLBERG, A. A.—(1934) *J. exp. Med.*, **60**, 199.

KEGEL, R. F. C.—(1932) *Arch. Surg.*, **25**, 498.

KRONFELD, R.—(1930) *J. Amer. dent. Ass.*, **17**, 681.

ORBAN, B.—(1949) 'Oral Histology and Embryology.' London (Kimpton).

ROBINSON, H. B. G.—(1937*a*) *Arch. Path.*, **23**, 664.—(1937*b*) *Ibid.*, **23**, 831.

SCHROFF, J.—(1931) *J. dent. Res.*, **11**, 635.

SIEGMUND, H.—(1929) *Fortschr. Zahnheilk.*, **5**, 243.

Idem AND WEBER, R.—(1926) 'Pathologische Histologie der Mundhöhle.' Leipzig (Hirzel).

SIMMONS, C. C.—(1928) *Ann. Surg.*, **88**, 693.

SPRAWSON, E. C.—(1937) *Brit. dent. J.*, **62**, 177.

Idem AND KEIZER, W. R.—(1933) *Dent. Rec.*, **53**, 369.

STONES, H. H.—(1948) 'Oral and Dental Diseases.' Edinburgh (Livingstone).

THOMA, K. H.—(1948) 'Oral Surgery.' London (Kimpton).—(1950) 'Oral Pathology.' London (Kimpton).

Idem AND GOLDMAN, H. M.—(1946) *Amer. J. Path.*, **22**, 433.

Idem AND PROCTOR, C. M.—(1937) *Int. J. Orthod.*, **23**, 307.

TOLLER, P. A.—(1948) *Proc. Roy. Soc. Med.*, **41**, 681.

WATERWORTH, G. E., AND PULLAR, T. H.—(1948) *J. Path. Bact.*, **60**, 193.

WILLIS, R. A.—(1948) 'Pathology of Tumours.' London (Butterworth).

STUDIES IN EXPERIMENTAL GOITRE: MALIGNANT CHANGE IN A TRANSPLANTABLE RAT THYROID TUMOUR.

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PREVIOUS communications from this laboratory (Griesbach, Kennedy and Purves, 1945; Purves and Griesbach, 1946, 1947) described the induction of thyroid adenomata in rats by the long-term administration of goitrogens. Bielschowsky, Griesbach, Hall, Kennedy and Purves (1949) showed that these thyroid tumours as well as those appearing under the combined influence of goitrogen and carcinogen could be transplanted into rats of the same strain. Transplantation was only successful when the recipient animal was kept in a state of thyroxine deficiency produced either by treatment with goitrogen or thyroidectomy. From this failure of the transplanted tissue to grow in normal animals it was concluded that an excess of thyrotrophin was needed for the continued progressive growth of such tissue in a similar way as it had been necessary for the induction of the original tumour in the rat's thyroid. Therefore, these tumours were not considered to be "autonomous," although they showed such signs of malignancy as a typical and progressive growth, invasion of surrounding tissues and formation of metastases. The transplants grew rather slowly and never seemed to cause the death of the host. The tumour, therefore, was considered to be of a low grade of malignancy.

Experimental tumours are generally considered to undergo increases in malignancy on transplantation, though there is doubt as to whether these increases are due to gradual adaptation to the host or to mutations in the tumour tissue giving rise to more malignant variants which are selectively propagated under the conditions of serial transplantation. This report describes changes occurring in the thyroid tumour strain T_1 during 3 years of propagation by serial transplantation. These observations have a bearing on the problem of malignant changes in transplanted tumours.

METHODS.

The strain of rats used was the same as described before (Griesbach, Kennedy and Purves, 1945; Purves and Griesbach, 1946, 1947). Transplantation of the tumour tissue was made by implanting the small pieces subcutaneously under the skin of young rats by the use of a small trocar. In some experiments the tumour tissue was finely minced with a small mincer and the mass injected by the Bashford syringe, 0·05 ml. being injected subcutaneously in the flank of each rat. The majority of rats were continuously supplied with a ·01 per cent solution of methylthiouracil as drinking water. Special mention is made in the text when no goitrogen was given. Some animals were treated with thyroxine, receiving daily injections of 10 μg. of DL thyroxine in 1 ml. of normal saline.

RESULTS.

Origin of Tumour T_1.

The tumour strain with which this communication is concerned was obtained from a male rat which from June, 1946, was given 0·01 per cent of methylthiouracil in the drinking water. In May, 1948, the rat was anaesthetized with ether and the thyroid inspected. The thyroid was very large with a nodule protruding from the isthmus. Part of this nodule was removed and two pieces were inoculated into two young rats which had been given 0·01 per cent methylthiouracil beginning 5 days previously. The original rat was then treated with thyroid substance, and at later examination the thyroid and the tumour which had been left were found to have regressed in size. Both the grafted rats developed tumours, these forming visible nodules (first generation grafts). Fig. 2 and 3 show the appearance of one of these rats 5 months after the initial grafting.

Histology of Tumour T_1.

Histological material from the first generation grafts showed that both animals had the same type of tumour. The tumour was an adenoma with well-developed acinar structure. The acini varied in size, many of them being larger than the acini of the normal rat thyroid. In one line of transplantation this structure has been maintained up to the present day, and Fig. 6 shows the histology of a graft from the fourth generation of this line. It will be noted that surrounding the larger acini there are numbers of small acini which suggest that new acini are being formed by budding from the larger ones. Small cellular areas exist without well-defined acini, but these are considered to be areas of proliferation in which differentiation has not yet taken place. The nuclei are large and hyperchromatic and are closely crowded in the walls of the acini. The acini are well filled with colloid, this colloid accumulation being always present even under conditions of strong thyrotrophic stimulation. This presence of colloid does not, however, indicate that the tumours are not influenced by thyrotrophic hormone, since when the thyrotrophic hormone production in the host is inhibited, these tumours undergo rapid regression.

Iodine metabolism of T_1 tumour.

The iodine metabolism of the transplanted T_1 tumour was tested in rats bearing second generation grafts. Table I shows the results of *in vivo* tests of the uptake of radioactive iodine in three rats after radioactive iodine had been injected. The measurements were made by positioning the rats so that the rats' thyroid or the grafted tumour was opposite a small hole in a lead plate, behind which was situated a Geiger counter connected to a conventional scaling apparatus. The results indicate that the transplanted tumours had an iodine metabolism quantitatively equal to that of the animals' own thyroid. Concentration of the iodine and assimilation into organic form occurred despite the administration of 0·01 per cent methylthiouracil in the drinking water. Later experiments showed that 0·05 per cent methylthiouracil in the drinking water would inhibit the assimilation of radioactive iodine into organic form in both the normal thyroid and the transplanted tumour. In animals receiving 0·01 per cent methylthiouracil the formation of organic iodine compounds by the thyroid is hindered but not entirely inhibited. In these animals, therefore, a state of partial thyroxine deficiency exists.

Bearing in mind the relative size of the tumour graft and the hyperplastic thyroid in these animals, it is considered that the iodine metabolism of the T_1 tumour is about one-fifth that of normal rat thyroid. This result has been confirmed in later experiments in which the iodine concentration of the excised tumour tissue has been measured and compared with that of the thyroid of the same animal.

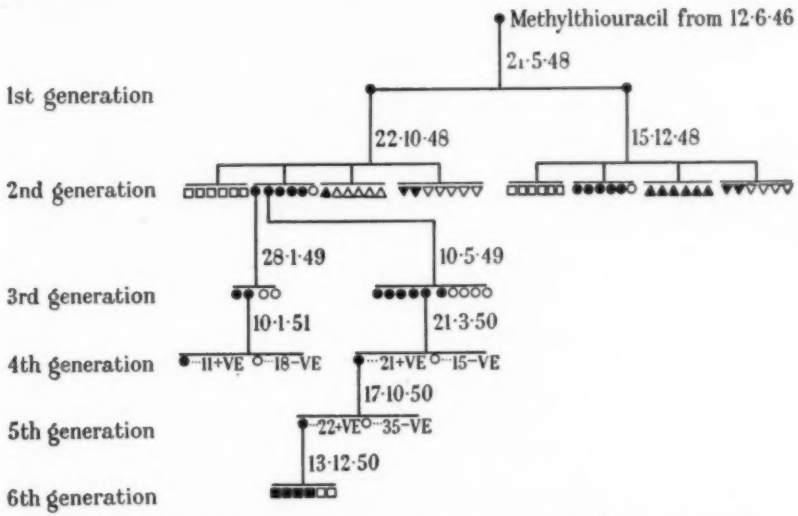


FIG. 1.—Chart of the first 6 generations' grafting of the thyroid Tumour T_1 The treatment of the recipient animals is indicated on the chart. Solid black indicates animal bearing tumour. The date of grafting is indicated opposite the vertical lines.

□ = Normal. ○ = Methylthiouracil. △ = Subtotal thyroidectomy. ▽ = Total thyroidectomy.

TABLE I.—*Accumulation of* I^{131} *in Tumours and Thyroids of Rats with Transplanted* T_1 *Tumour.*

No. of rat.	Treatment.	Counts per minute per μc. of I^{131} injected.			
		1 hr. after injection.		20 hr. after injection.	
		Tumour.	Thyroid.	Tumour.	Thyroid.
1	Methylthiouracil 0·1% in the drinking water	48	65	112	55
2	Ditto	86	25	30	38
3	Methylthiouracil stopped 5 days previously	54	74	89	93
4	Ditto	66	62	80	76
5	,,	30	44	46	101

The rats were injected with varying doses of I^{131} ranging from 75 μc. to 300 μc. The counts from the tumour and the thyroid regions have been corrected for the effect of circulating I^{131} by subtracting the counts obtained from the thigh to obtain net counts for tumour and thyroid.

The evidence of secretory function in the T_1 tumour has been obtained from the results of transplants growing in totally thyroidectomized animals. In these animals the T_1 tumour transplants do not grow continually, but appear to reach a maximum weight of about 1 g. The histological examination of the pituitary of such animals shows the effectiveness of the secretion of the T_1 tumour in repairing the thyroxine deficiency which would otherwise exist in the thyroidectomized animal. Table II shows the distribution of the cell types in

TABLE II.—*Influence of Tumour Secretion on Pituitary Cell Composition.*

State of animal.	No. of animals.	Pituitary.		
		Acidophils (%).	Basophils (%).	Chromophobes (%).
Normal	10	51·2	8·7	40·1
Thyroidectomy, without graft	4	0·0	13·3	86·7
Thyroidectomy,* with graft	3	63·5	11·1	25·4

* Completeness of thyroidectomy checked by tracheal sections.

the pituitary of such animals as compared with totally thyroidectomized animals not bearing tumour grafts and normal animals. The repair of the acidophil cells to normal proportions and the reduction in the number of basophils indicate that the amount of thyroxine produced by such tumours is almost sufficient to

EXPLANATION OF PLATES.

FIG. 2.—Tumour graft on right side of Rat 126, No. 2, 5 months after transplantation. The animal was given 0·01 per cent methylthiouracil in the drinking water.

FIG. 3.—Tumour graft on left side of Rat 126, No. 2. This tumour was removed after the photograph was taken and used for grafting experiments.

FIG. 4.—Tumour graft on right side of Rat 126, No. 2, 14 days after Fig. 1 was taken. The rat received no methylthiouracil in the interim period. The tumour has regressed considerably and was still shrinking rapidly.

FIG. 5.—Rat 253, No. 2, showing 107 g. tumour in fourth generation graft. The weight of the rat after removal of the tumour was 84 g.

FIG. 6.—Histology of T_1 tumour in the fourth generation graft of line B. This structure is identical with that observed in the first generation grafts. H. & E. × 95.

FIG. 7.—Radio-autograph of T_1 tumour with superimposed section, with nuclear staining by neutral red. The silver grains show accumulation of radio-active iodine in the colloid. Neutral red. × 95.

FIG. 8.—Section of grafted tumour mass of Rat 253, No. 1 (T_1 tumour graft, fourth generation, line A). The upper portion shows a microfollicular adenoma different from Fig. 6. The lower portion shows an anaplastic growth which is encapsulated (T_{22}). H. & E. × 95.

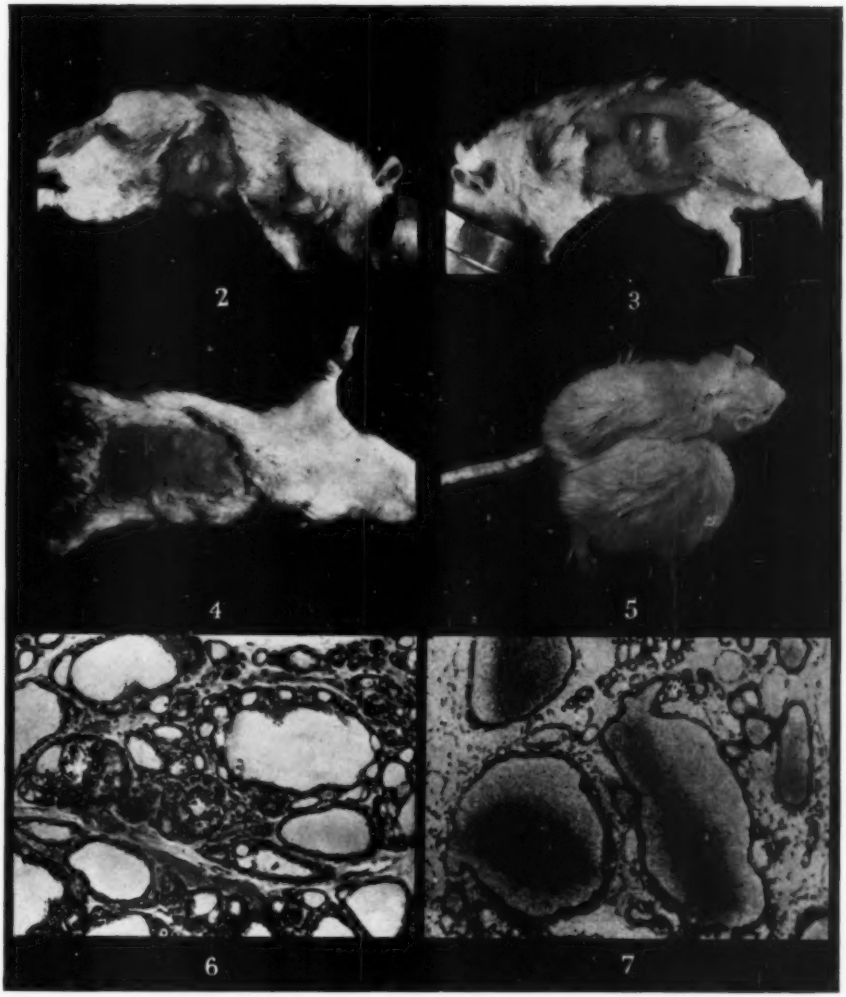
FIG. 9.—High-power view of the anaplastic tissue (T_{22}) of Fig. 8. There is a great variation of nuclear size, many large cells with large nuclei being present. Mitoses are frequent. H. & E. × 450.

FIG. 10.—Section of grafted tumour of Rat 253, No 2 (T_1 tumour, fourth generation graft, line A). Both the microfollicular adenoma and the anaplastic growth, seen in Rat 253, No. 1 (Fig. 8), are present in this section, but the anaplastic tissue is not encapsulated and is invading the adenoma tissue. H. & E. × 95.

FIG. 11.—Section of another part of transplanted tumour in Rat 253, No. 2, showing small nodule of lightly staining tissue closely resembling normal hyperplastic thyroid. H. & E. × 95.

FIG. 12.—High-power views of section of transplanted T_{22} tumour, showing (*a*) a giant cell and (*b*) an atypical mitosis. H. & E. × 450.

FIG. 13.—Section of lung of rat, killed 6 weeks after inoculation with T_{22} tumour. A metastasis is shown which has penetrated the elastic tissue of the pleura and formed a nodule on the surface of the lung. Gomori elastic tissue stain, H. & E. × 95.



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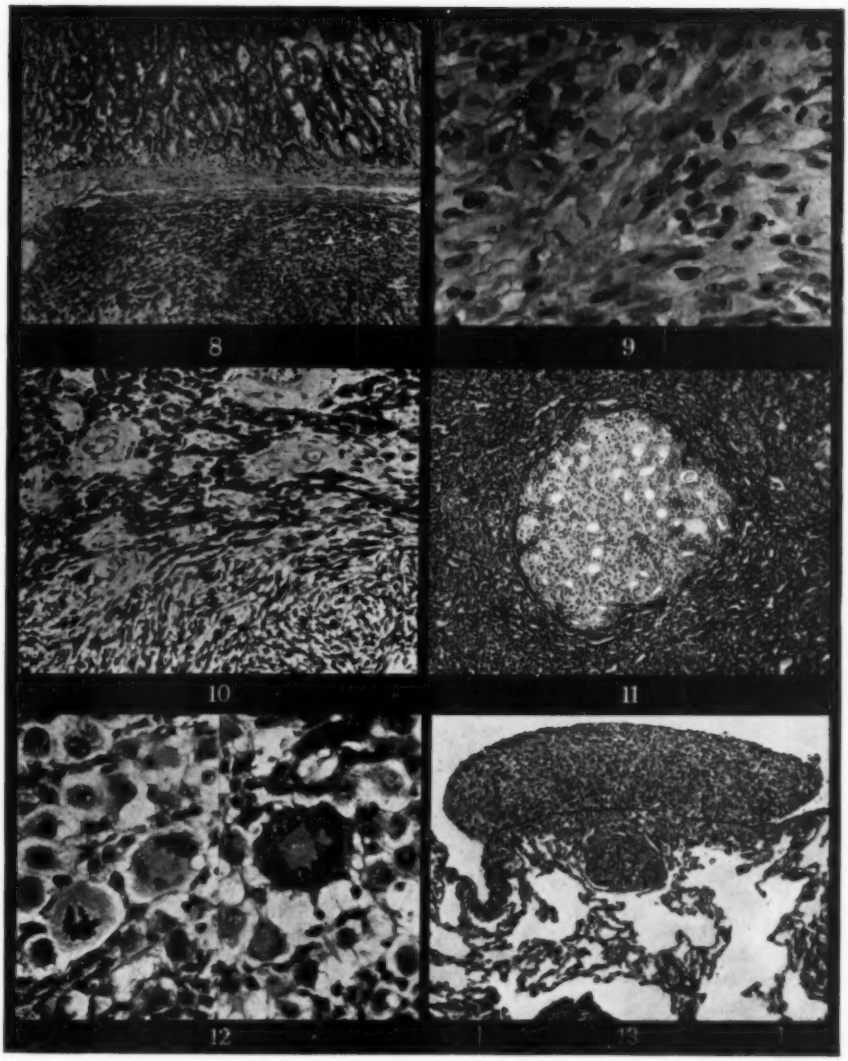

Purves, Griesbach and Kennedy.

meet the requirements of the animal. Some residual thyroxine deficiency exists under these conditions, since it is only when the thyrotrophic hormone is unnaturally high that this T_1 tumour maintains itself.

Dependence of the Tumour T_1 on thyrotrophic hormone.

In the second generation, grafting of the tumour experiments were made to determine the conditions necessary for the continued growth of this tumour. Inoculations were made into 4 groups of rats as under :

(*a*) rats on stock diet without special treatment ;
(*b*) rats receiving methylthiouracil in the drinking water ;
(*c*) rats sub-totally thyroidectomized, leaving a small portion of the isthmus in place ;
(*d*) rats totally thyroidectomized.

The results are summarized in Table III. It will be seen that continued growth of the grafts was obtained in the three states of thyroxine deficiency, while no

TABLE III.—*Results of Transplantation Experiments in the Second Generation Transplantation of Tumour T_1.*

Condition of rats.	No. of rats inoculated.	No. of rats with tumour growth.
Normal	12	0
Methyl thiouracil 0·1% in the drinking water	12	10
Subtotally thyroidectomized . . .	11	7
Totally thyroidectomized, ...	13	4

growth was obtained in the normal animals. This indicates the dependence on high thyrotrophic hormone levels, a dependence which is also displayed when animals bearing growing tumours during the administration of methylthiouracil have their medication stopped. Under these conditions the tumours rapidly regress, and after 6 months the tumours may have entirely disappeared and do not reappear when the animals are again treated with methylthiouracil.

Structures visible in the fourth generation transplants.

In the fourth generation transplants of line A (Fig. 1) 3 different types of histological structure were observed, none of them corresponding to the original adenoma.

(1) The bulk of the tumour growing from the graft was a microfollicular adenoma. The acini showed variation in size from minute up to about 15μ diameter. The large irregularly-shaped acini which were prominent in the T_1 tumour were not seen. Colloid was present in some of the acini but was less prominent than in the T_1 tumour. The nuclei were hyperchromatic and irregular in shape.

(2) Embedded in this adenomatous tissue was a nodule of tissue distinctly different in staining properties. In this tissue, which had a well-developed acinar structure corresponding to a very hyperplastic normal rat thyroid, the cells were large with abundant cytoplasm. The nuclei were round and did not contain excess chromatin. The lesser numbers of nuclei and their weaker staining properties were responsible for the paler appearance of these nodules in the

stained section, causing the nodule to stand out from the group of hyperchromatic nuclei of the adenomatous tissue (Fig. 11). This type of tissue was seen in each of the 2 animals mentioned above and was also seen in one of the grafts of the first generation. The fact that such tissue always forms microscopic nodules and has not ever formed a large part of the grafts in any of the inoculated animals suggests that this type of tissue is a slow-growing variant which arises spontaneously within the adenomatous tissue. The nodules of this type seen in the fourth generation appear to have arisen from the modified adenomatous tissue of these animals mentioned above which differs from that of the original tumour.

(3) The third type of tissue is an anaplastic carcinomatous tissue without any acinar structure. In Animal 253, No. 1, the anaplastic tissue is seen as a discrete mass adjacent to masses of the adenoma but separated from them by fibrous septa. In Animal 253, No. 2, this anaplastic tissue is seen infiltrating and replacing the adenomatous structure without a sharp demarcation (Fig. 8, 10).

Growth of transplanted Tumour T_{22}.

Tumour tissue from one of the animals (253, No. 1) was minced and injected into 57 young rats which were given 0·01 per cent methylthiouracil. Growth of the transplanted tumour was obtained in 22 of these rats. The growth was much more rapid in this generation than had ever been observed before, tumours of about 1 g. in weight being observed after 10 days. In the fourth week after transplantation animals with the rapidly growing tumours began to succumb. In a proportion of the animals, however, the growth was slower and the animals survived 3 or 4 months. The histology of all these tumours was that of the anaplastic carcinoma described above as the third type of tissue seen in the fourth generation. This tumour, now called T_{22}, was successfully maintained by serial transplantation, and in later transplantations successful grafts were obtained in up to 80 per cent of those inoculated. The rate of growth in different animals continued to show a wide range of variation, but was always much more rapid than was obtained with the T_1 tumour. The T_{22} tumour has now been transplanted through 7 consecutive generations and has not undergone any progressive change in either malignancy or histology.

Histology of Tumour T_{22}.

On the cut surface two regions could be regularly seen, the outer with a shiny grey appearance, 5 to 8 mm. broad, and the central one coloured a pale orange with a crumbled surface. Frequently a brown albuminuous fluid had replaced the central tissue. Microscopic examination revealed that, generally, the inner zone of the tumours consisted of degenerating cells or amorphous masses, infiltrated by round cells, and isolated cells with strongly eosinophilic cytoplasm and pycnotic nucleus, lying between the cell debris.

The outer zone, microscopically, appeared similar to that of the anaplastic nodule of the original tumour, and this description applies to both tissues. They consisted of densely crowded cells the majority of which were spindle-shaped, while others were round or of quite irregular shape. Their size varied considerably. There was no proper stroma of the tumour recognizable. Only a few coarse collagen fibres were to be found between the tumour cells. The number of blood-vessels was extremely small. In some parts of the tumour the cells were arranged in strands, with a tendency to form whorls. In other parts of the nodule

no special pattern was recognizable. The nuclei also showed considerable variations in shape, size and chromatin content. The smaller elements had dense nuclei, while the larger cells had vesicular nuclei with one, rarely two, rather prominent nucleoli. Unusually large tumour cells were present which had slightly basophilic cytoplasm and nuclei with irregularly dispersed chromatin. Giant cells were present with nuclei varying in number from 2 to 8. The numbers of giant cells found varied somewhat in different animals and were relatively few in the first animal examined. They have, however, been a constant feature of the tumour, and we consider that the tumour should, therefore, be classed as a giant cell carcinoma of the thyroid. Mitotic figures are frequent and many of them atypical.

Effect of T_{22} tumour on the host.

The striking feature of the T_{22} tumour is that it kills its host in as short a period as 3 weeks. There is, however, a considerable variation in the rate of growth in different animals, some of them surviving for 2 months or more. In those animals which died within 3 to 4 weeks after inoculation, the animals showed at autopsy an extreme state of emaciation with atrophic muscles and thin soft bones. The tumours' weights varied between 30 and 50 g. After removal of the tumours the carcass weight of the animals was lower than its weight at the time of transplantation. It appears, therefore, that the rapid growth of these tumours withdraws nutrients from the host and leads to the state of emaciation described.

Effect of thyrotrophic hormone level on Tumour T_{22}.

The T_{22} tumour has been found to grow equally well when inoculated into normal animals or animals receiving methylthiouracil. Moreover, in normal animals in which this tumour is growing, no inhibition of the growth rate has been observed when the animals were treated with thyroxine injections. In some selected animals bearing this tumour in which the growth was relatively slow some were treated with thiouracil. This treatment did not produce any marked acceleration in the growth of these tumours. The T_{22} tumour, therefore, does not show the dependence upon thyrotrophic stimulation which was shown by its parent tumour.

Iodine metabolism of the T_{22} tumour.

Rats bearing the T_{22} tumour were injected with radio-active iodine and 24 hours later were killed, and the radio-active iodine concentrations in the tumour tissue and in the blood plasma were measured. The radio-active iodine concentration of the tumour tissue was approximately 50 per cent of that in the plasma. These tumours, therefore, show no selective iodine concentration, a finding which is in agreement with the anaplastic nature of the tumour. Clinical experience shows that iodine concentration is to be expected only in those tumours in which an acinar structure is present.

DISCUSSION.

The role of goitrogens in the production of primary tumours in the thyroid.

When Bielschowsky (1944) described the production of thyroid tumours in the rat by combined treatment with allylthiourea and acetamidofluorene he

considered that both the action of the carcinogen plus the hyperplasia resulting from the goitrogen were necessary for the induction of thyroid tumours. However, Griesbach, Kennedy and Purves (1945) reported that the prolonged action of goitrogens alone led to the formation of tumours, which in a later publication (Bielschowsky, Griesbach, Hall, Kennedy and Purves, 1949) were shown to be similar in all respects to those appearing after the carcinogen treatment. With goitrogen alone, however, tumours are later in appearing and are fewer in number. These tumours are dependent upon high thyrotrophic hormone level for their growth. In normal animals such tumours, either in the thyroid or as grafted tumours, undergo rapid regression and eventually disappear, so that they cannot be induced to reappear by the subsequent administration of goitrogen. The simplest explanation, therefore, of the role of the goitrogen in the production of these tumours is that it induces the high thyrotrophic hormone production without which such tumours cannot grow. In this view, the goitrogen would not have any influence on the formation of neoplastic cells. It is, therefore, considered that neoplastic cells arise spontaneously in the normal rat thyroid, and that when conditions are suitable for the growth of such cells, visible neoplasms result. The carcinogen seems to act by speeding up the formation of neoplastic cells. The non-occurrence of thyroid neoplasms in normal rats is explained by the fact that all such primary thyroid tumours in the rat require high thyrotrophic hormone levels for their growth.

Mutations in transplanted adenomas.

Since the primary adenomas of the rat thyroid strongly resemble the normal thyroid tissue it is not surprising that there should arise within them areas of tissue differing in structure from the parent adenoma. Presumably these areas arise from changes in single cells of the adenoma similar to the original change which produced the adenoma from the normal thyroid cell. The observation of such changes is facilitated by reason of the large mass of thyroid adenoma that can be maintained, since tumours up to 5 g. in weight are commonly produced. The continuous growth of such tumours and the propagation of them by transplantation leads naturally to the selection of fast-growing types of tissue so that an increase in malignancy with transplantation is to be expected. However, modifications of the adenoma are not invariably in the direction of increased malignancy. We consider that the type of nodule described as the second type of tissue observed in the fourth generation transplants is a relatively slow-growing benign structure which reappears in successive generations, and which by virtue of its slow growth and lack of metastasizing power cannot be selectively propagated by transplantation. On the other hand, the truly malignant, invasive and metastasizing carcinoma will, when it once appears, invariably supplant entirely the benign structure in two, or three at the most, generations of grafting.

Evolution of malignancy in benign tumours.

While it has been recognized that transplantable mammalian tumours show increases in malignancy on transplantation, it has been often assumed that such increase in malignancy results from a gradual adaptation to the host or a gradual modification of the original tumour. The well-defined structure of the thyroid adenomata, however, makes it easy to see that modifications of the original tumour arise by distinct mutations and not by gradual changes. Thus, in the

parallel line of the T_1 tumour strain in which at this time the original structure is still maintained there has been no increase in growth rate or malignant behaviour. Such changes in rate of growth and in malignancy as have been observed in the material at present described have been accompanied by and are due to the formation of a new type of tumour. It is considered that mutations of this sort are of sufficiently frequent occurrence to account for changes in tumour behaviour on transplantation.

It seems important to note that malignant anaplastic tumours similar to the T_{22} have not been seen in over 100 rats which have been examined with primary thyroid tumours. All of these tumours have been of the adenomatous type with well-defined acinar structure. This is true, too, of all the tumours seen after administration of the carcinogen, acetamidofluorene. It therefore seems that the anaplastic tumour is derived from the original thyroid cell by more than one (in this case apparently 3) distinct steps. This observation fits well with the clinical observation that malignant changes in benign tumours are of frequent occurrence, but goes further in suggesting, at least as regards the rat thyroid, malignant tumours can be formed only from benign tumours. It may be found that such a mechanism for the production of malignant tumours may be of more frequent occurrence in human material than is at present realized, since the benign tumour from which the malignant tumour is derived, although it may have existed for a long time, may be quite small and may, therefore, easily escape detection, either at *post mortem* or operation. Furthermore, the benign tumour may not differ very much from the normal tissue and may, therefore, not be recognized as a neoplasm. In this connection it should be noted that while adenomata in the rat thyroid have been observed by many people who have examined rats after long-term goitrogen administration, not all workers have recognized their neoplastic nature.

Autonomy of thyroid carcinomas.

It has been considered without the support of experimental evidence that one of the features of neoplasms is that they are not subject to the controlling influences which regulate the growth of normal tissue. However, this hypothesis is one which cannot be tested unless all the influences which regulate growth in normal tissue are first known. Only then is it possible to test experimentally whether the neoplasms are in fact independent of the growth stimuli which normal tissues require. The thyroid adenomata appearing in the rat thyroid are all dependent on thyrotrophic hormone for their existence, and in fact require higher levels of thyrotrophic hormone for their continued growth than does the normal thyroid tissue. Investigation of human thyroid neoplasms with the aid of radio-active iodine have shown that many of these are in fact susceptible to the stimulating influence of thyrotrophic hormone, although neoplasms in human material have not been described which are so entirely dependent upon thyrotrophic hormones as are these primary rat thyroid tumours. The existence of undoubted stimulating effects of thyrotrophic hormone in human thyroid tumours supports the view that these rat thyroid tumours are not exceptional, although their high degree of dependence on thyrotrophic hormone sets them somewhat apart from the thyroid tumours encountered clinically. There seems no reason to hold the view, as some people have done, that these rat tumours should not be classed as neoplasms, since in their case the stimulating hormone or factor on which their

growth depends is well characterized and can be artificially manipulated so as to control the tumour growth. Presumably as further knowledge is gained the stimulating influences which condition the growth of other tumours will be discovered, but this should in no way affect their classification as neoplasms.

It is important to note here that from a tumour at first dependent upon a hormonal imbalance for its growth, there has been derived a malignant neoplasm which is no longer dependent upon the stimulating influences which condition the growth of the more benign original tumour. Thus where malignant tumours are found which are not susceptible to any known hormonal influence, the effect of hormonal imbalance in the production of such tumours is not excluded. The existence of hormonal imbalance over a period of years may provide the stimulus to the growth of a primary benign tumour from which a malignant variant is derived, which itself shows no dependence upon hormonal stimulus. This result may have important clinical application if it becomes possible to recognize pre-cancerous states which can be controlled by the variation of hormonal levels, since it suggests a way in which the appearance of malignant tumours might be prevented by appropriate treatment of pre-cancerous states.

SUMMARY.

The behaviour of a rat thyroid tumour appearing during long-term methyl-thiouracil administration is reported. The tumour was successfully transplanted into rats with thyroxine deficiency. It underwent changes during serial transplantation, three different types of histological structure being produced from the original tumour. One of these structures was a malignant anaplastic carcinoma which was transplantable in rats without thyroxine deficiency.

It is concluded that while primary tumours of the rat thyroid all require high thyrotrophic hormone levels for their growth, tumours of a more malignant character, not influenced by thyrothrophic hormone, may appear by malignant change in the original adenomata. These results have a possible bearing on the prevention of malignancy by the adequate treatment of pre-cancerous states.

REFERENCES.

Bielschowsky, F.—(1944) *Brit. J. exp. Path.*, **25**, 90.

Idem, Griesbach, W. E., Hall, W. H., Kennedy, T. H., and Purves H. D.—(1949) *Brit. J. Cancer*, **3**, 541.

Griesbach, W. E., Kennedy, T. H., and Purves, H. D.—(1945) *Brit. J. exp. Path.*, **26**, 18.

Purves, H. D., and Griesbach, W. E.—(1946) *Ibid.*, **27**, 294.—(1947) *Ibid.*, **28**, 46.

EFFECT OF ADENOSINE-5-PHOSPHORIC ACID, ADENOSINE-3-PHOSPHORIC ACID AND ADENOSINE TRIPHOSPHATE ON THE GROWTH OF EHRLICH CARCINOMA.

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CASPERSSON and his associates advanced a hypothesis on the role of nucleic acids in the biological synthesis of proteins (Caspersson, 1947). Several other results suggested the role of nucleotides in the metabolism of tumors as well, as a result of which many structural analogues of pyrimidine and purine derivatives were synthesized and tested for anti-cancer and growth-inhibiting activity (Roblin, Lampen, English, Cole and Vaughan, 1945; Kidder and Dewey, 1949; Kidder, Dewey, Parks and Woodside, 1949; Sugiura, Hitchings, Cavalieri and Stock, 1950; Burchenal, Bendich, Brown, Clian, Hitchings, Rhoads and Stock, 1949; Skipper, Bennet, Edwards, Bryan, Hutchison, Chapman and Bell, 1950; Law, 1950; Lewis and Crossley, 1950.) However, little is known about the effect of the naturally occurring nucleotides on tumor growth.

Parsons, Gulland and Barker (1946, 1947) described that in C57 mice the growth of homologous methylcholanthrene sarcoma grafts was inhibited by (yeast) adenylic acid or guanylic acid; uridylic acid showed a growth-promoting effect, whereas cytidylic acid had no effect. The excellent review of Dyer (1949) covers the publications of some earlier authors, investigating the effect of several nucleotides on tumor growth.

METHODS.

Since it was expected that the nucleotides might cause relatively small changes in the rate of tumor growth, sensitive methods were sought. Ehrlich ascites tumor was used; reviews on this tumor were given by Lettré (1941), and more recently by Klein (1950), and Klein and Klein (1951). The tumor was carried in the abdominal cavity of Strong A mice. Tumor cells are free in the ascitic fluid, usually without forming solid tumors. Ascitic fluid was harvested by abdominal puncture under aseptic conditions, and the number of cells present in the ascitic fluid counted in a hemacytometer. The percentage of inflammatory cells was established by a smear, stained according to the method of Papanicolaou (Papanicolaou and Traut, 1943). From these data the number of tumor cells in 1 c.mm. of ascitic fluid was calculated, and inoculations were made aseptically with known numbers of cancer cells. Within any one series the control as well as the experimental groups were inoculated with cancer cells originating from the same donor.

The method used in this study offers the following advantages: (*a*) cancer cells of known number and equal virulence are inoculated into the control, as well as the experimental animals; (*b*) upon intraperitoneal inoculation similar ascitic tumors are produced, while subcutaneous inoculation results in the development of solid tumors; (*c*) in case of intraperitoneal inoculation the action of substances upon the cancer cells themselves may be tested without involving such factors as tumor vascularization, differences in distribution of the injected substances, etc. The error in this method is that there is always some microscopic infiltration of solid tissues in the peritoneal cavity (Klein, 1950; Klein and Klein, 1951). These infiltrating cells are, of course, not included in our cell counts.

Swiss mice, bred in our colony, were used for these studies. These mice were maintained on Rockland Complete Rat Diet and water, *ad libitum*, and kept in air-conditioned quarters at 21° C. They were weighed daily. These mice were greatly susceptible to the cancer cells originating from Strong A mice in which the stock tumor was carried. The strain aspecificity of this tumor was discussed by Klein (1950) and Klein and Klein (1951). Subcutaneous inoculations were made into the caudal third of the back. Twenty-one days after inoculation the animals were sacrificed, and their tumors dissected and weighed. Groups receiving intraperitoneal injections of cancer cells were observed for mortailty rate. Animals dying were autopsied, their ascitic fluid harvested and measured, and the number of cancer cells per cubic millimetre established as previously described.

Nucleotides were administered by daily subcutaneous injection, alternating between the right and left flank. Adenosine-3-phosphoric acid was injected in solution in 3·2 per cent disodium phosphate, adenosine triphosphate and adenosine-5-phosphoric acid in normal saline warmed to about 37° C. The control group in Series I and V received corresponding amounts of disodium phosphate, while the control groups in Series II, III, IV and VI were injected with similar volumes of normal saline.

RESULTS.

According to preliminary experiments the maximal dose of the nucleotides which could be administered by subcutaneous injection daily without evident toxic effect to Swiss mice during a period of 6 weeks was as follows: adenosine triphosphate, 100 mg./kg.; adenosine-5-phosphoric acid, 500 mg./kg.; and adenosine-3-phosphoric acid, 500 mg./kg. These doses were, therefore, used in all the final experiments.

Subcutaneous inoculations.

In the first three series 2,000,000 cancer cells were inoculated while the fourth series received 200,000 cells. The mean tumor weights 3 weeks after inoculation are shown in Table I. Adenosine-3-phosphoric acid exhibits a definite tumor-inhibiting effect. Adenosine-5-phosphoric acid seems rather to enhance tumor growth; this may be more readily evidenced in the series using a smaller number of inoculated cells. Adenosine triphosphate has no effect.

Intraperitoneal inoculations.

In Series V 1,000,000 cancer cells were inoculated intraperitoneally into 3 groups of 15 mice each. Mortality curves are shown in Fig. 1. Adenosine

triphosphate as well as adenosine-5-phosphoric acid increases the death rate of animals with ascitic tumors. In Series VI 10,000,000 cancer cells were inoculated into groups of 15 mice. The usual doses of nucleotides were used, and one additional group received 10 mg./kg. adenosine triphosphate. The results are shown in Fig. 2. All nucleotides increase death rate of tumor-bearing animals.

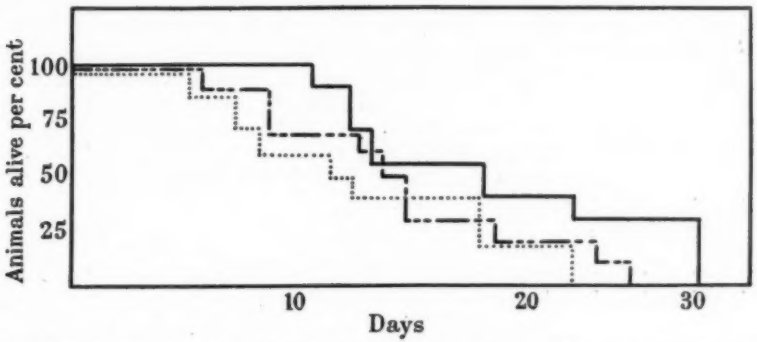

FIG. 1.—Effect of nucleotides on the death rate of mice inoculated intraperitoneally with 1,000,000 Ehrlich carcinoma cells (Series V). Treatments: Control ———; adenosine triphosphate 100 mg./kg. .....; adenosine-3-phosphoric acid 500 mg./kg.— - - —

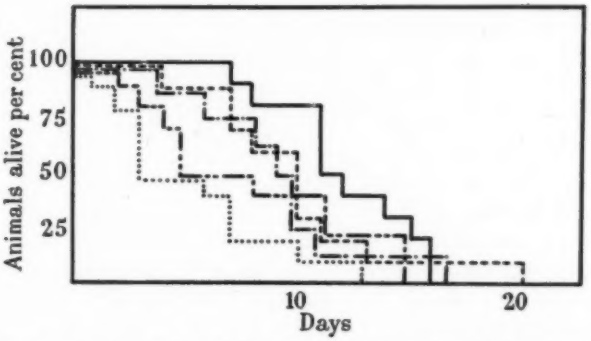

FIG. 2.—Effect of nucleotides on the death rate of mice inoculated intraperitoneally with 10,000,000 Ehrlich carcinoma cells (Series VI). Treatments: Control ———; adenosine triphosphate 10 mg./kg. — - —; adenosine triphosphate 100 mg./kg. .....; adenosine-3-phosphoric acid 500 mg./kg. - - - - - -; adenosine-5-phosphoric acid 500 mg./kg.

Mice bearing subcutaneous tumors lost weight progressively, but no significant difference was evidenced in this respect between control and nucleotide-treated animals. Nucleotide-treated non-tumor-bearing animals exhibited no weight loss. The weight of intraperitoneally inoculated mice increased gradually with the development of the ascites. This increase was somewhat more expressed in the control than in the treated groups, the difference being, however, statistically not significant.

TABLE I.—*Groups Treated with Different Nucleotides. Mean Tumor Weights in Grams.*

	Control.	Adenosine-3-phosphoric acid 500 mg./kg.	Adenosine-5-phosphoric acid 500 mg./kg.	Adenosine triphosphate 100 mg./kg.
Series I (2,000,000 cells):				
Mean	11·3	7·6	—	11·0
SE	1·0	0·895	—	1·193
N	15·0	15·0	—	15·0
Series II (2,000,000 cells):				
Mean	10·4	5·74	—	10·6
SE	1·56	1·05	—	0·94
N	10·0	10·0	—	9·0
Series III (2,000,000 cells):				
Mean	9·82	—	11·7	—
SE	1·48	—	1·73	—
N	11·0	—	12·0	—
Series IV (200,000 cells):				
Mean	0·96	—	1·7	1·05
SE	0·264	—	0·576	0·231
N	11·0	—	9·0	12·0

Autopsies revealed no unusual findings; no signs of concurrent infections were noted.

It is difficult to evaluate the results of the microscopic examination of the ascitic fluids from these mice since the individual animals died at different time intervals after inoculation. Nevertheless, it was noted that ascitic fluids from animals treated with nucleotides contained less cancer cells and more inflammatory cells than those from control animals. In a number of cases nucleotide-treated animals had no ascites at all or developed an intra-abdominal solid tumor which may be, according to Klein (1950) and Klein and Klein (1951), a sign of low tumor virulence. No such phenomenon was noted in control animals. These results suggest that nucleotides may decrease the virulence of intraperitoneally injected cancer cells, yet the nucleotides are more toxic to cancer-bearing mice than to normal animals.

DISCUSSION.

Greenberg (1949) demonstrated that adenosine-5-phosphoric acid inhibits anaerobic glycolysis of brain preparations, while adenosine-3-phosphoric acid and adenosine triphosphate have no such effect. Fischer, Wehmeier and Jüngling (1934) described the neuro-inductor effect of adenosine-5-phosphoric acid, while Suomalainen and Toivonen (1939) could not obtain similar results with adenosine-3-phosphoric acid. Green and Bullough (1950) showed that large doses of adenosine triphosphate greatly decrease the mitotic activity in the skin of mice. All these data suggested the investigation of the action of these nucleotides on tumor growth.

Mitchell (1942, 1943) pointed out that after X-ray or γ irradiation of rapidly growing cells, accumulation of nucleotides is evidenced in the cytoplasm. Loofbourow (1942) demonstrated a release of nucleotides from tissues exposed to ultraviolet irradiation. It is possible that release of nucleotides and their derivatives from malignant tissue during therapeutic irradiation, necrosis or mechanical destruction of the surrounding tissues plays a certain role in tumor growth.

In our experiments all three nucleotides increased the death rate in intraperitoneally inoculated animals. Microscopic examination, however, revealed some evidence for the possible inhibitory action of nucleotides on tumour growth. This latter effect might have been due in part to the morbid condition of the treated animals. Green and Stoner (1950) showed that certain strains of rats bearing transplanted dibenzanthracene sarcomata exhibit decreased adenosine triphosphate dephosphorylase activity of the liver. It is not impossible that this factor played a role in the increased nucleotide sensitivity of mice bearing Ehrlich ascitic tumors. No increased toxicity of nucleotides was evidenced in animals with subcutaneous tumors. In these groups adenosine-3-phosphoric acid significantly decreased tumor size, and adenosine-5-phosphoric acid had a slight growth-increasing effect, while adenosine triphosphate was ineffective. Similar differences between the biological actions of these nucleotides were also noted by other authors in different fields of investigation as mentioned above.

SUMMARY.

1. Adenosine-3-phosphoric acid inhibits growth of subcutaneous Ehrlich carcinoma. Adenosine-5-phosphoric acid rather enhances tumor growth, whereas adenosine triphosphate has no effect.

2. All three of the nucleotides brought about an increase in the death rate of animals inoculated intraperitoneally with Ehrlich carcinoma. The number of cancer cells, however, was smaller in the ascitic fluid of the nucleotide-treated mice than in the control animals.

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REFERENCES.

BUCRHENAL, J. H., BENDICH, A., BROWN, G. B., CLIAN, G. B., HITCHINGS, G. H., RHOADS, C. P., AND STOCK, C. CH.—(1949) *Cancer*, **2**, 119.

CASPERSSON, T.—(1947) *Symp. Soc. exp. Biol.*, **1**, 127.

DYER, H. M.—(1949) 'An Index of Tumour Chemotherapy.' Nat. Cancer Inst., Nat. Inst. Hlth.

FISCHER, F. G., WEHMEIER, E., AND JÜNGLING, L.—(1934) *Nachr. Ges. Wiss. Göttingen*, **9**, 394.

GREEN, H. N., AND BULLOUGH, W. S.—(1950) *Brit. J. exp. Path.*, **31**, 175.

Idem AND STONER, H. B.—(1950) ' Biological Actions of the Adenine Nucleotides.' London (H. K. Lewis & Co.), p. 180.

GREENBERG, G. R.—(1949) *J. Biol. Chem.*, **181**, 781.

KIDDER, G. W., AND DEWEY, V. C.—(1949) *Ibid.*, **178**, 383.

Iidem, PARKS, R. E., Jun., AND WOODSIDE, G. L.—(1949) *Science*, **109**, 51.

KLEIN, G.—(1950) *Cancer*, **3**, 1052.

Idem AND KLEIN, E.—(1951) *Cancer Res.* (in press).

LAW, L. W.—(1950) *Ibid.*, **10**, 186.

LETTRÉ, H.—(1941) *Z. phys. Chem.*, **268**, 59.

LEWIS, M. R., AND CROSSLEY, M. L.—(1950) *Arch. Biochem.*, **26**, 319.

LOOFBOUROW, J. R.—(1942) *Biochem. J.*, **36**, 631, 737.

MITCHELL, J. S.—(1942) *Brit. J. exp. Path.*, **23**, 285, 296, 309.—(1943) *Brit. J. Radiol.*, **6**, 339.

PAPANICOLAOU, G. N., AND TRAUT, H. F.—(1943) 'Diagnosis of Uterine Cancer by the Vaginal Smear.' New York (Commonwealth Fund).

PARSONS, L. D., GULLAND, J. M., AND BARKER, G. R.—(1946) *Nature*, **157**, 482.—(1947) *Symp. Soc. exp. Biol.*, **1**, 179.

ROBLIN, R. O., LAMPEN, J. O., ENGLISH, J. P., COLE, Q. P., AND VAUGHAN, J. R.—(1945) *J. Amer. chem. Soc.*, **67**, 290.

SKIPPER, H. E., BENNET, L. L., EDWARDS, P. C., BRYAN, C. E., HUTCHINSON, O. S., CHAPMAN, J. S., AND BELL, M.—(1950) *Cancer Res.*, **10**, 166.

SUGIURA, K., HITCHINGS, G. H., CAVALIERI, L. F., AND STOCK, C. CH.—(1950) *Ibid.*, **10**, 178.

SUOMALAINEN, P., AND TOIVONEN, S.—(1939) *Ann. Acad. Sci. Fenn. A.*, **53**, No. 6.

THE EFFECTS OF COLD AND DESICCATION ON THE TUMOUR-PRODUCING ACTIVITY OF THE CELLS AND THE VIRUS OF THE ROUS NO. 1 FOWL SARCOMA.*

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INTEREST in the effects of cold and desiccation on normal and neoplastic tissues was recently stimulated by the work of Gye and his colleagues (Gye, Begg, Mann and Craigie, 1949; Mann, 1949*a*, 1949*b*, 1949*c*). These investigators showed that embryonic mouse tissue which normally grew when transplanted did not do so after freezing to a temperature of − 79° C. (Mann, 1949*a*), whereas a chemically induced and two sporadic mouse sarcomata were transmissible following freezing to − 79° C. and storing at that temperature and after drying from the frozen state (Gye, Begg, Mann and Craigie, 1949). In addition, mouse breast carcinoma which had been frozen to −79° C. gave rise to tumours when inoculated into mice (Mann, 1949*b*, 1949*c*). From these results it was concluded that since the physical treatments killed the normal embryo cells, they must also have killed the tumour cells, and that the tumours which arose from the inoculations of treated material must have been caused by a virus liberated from the dead cells.

The claims of Gye and his co-workers have been much criticized; Hirschberg and Rusch (1950), Passey and Dmochowski (1950), Passey, Dmochowski, Lasnitzki and Millard (1950) and Warner and Gostling (1950) have all pointed out the great weakness of the assumption that since embryonic cells failed to grow after exposure to low temperature, such treatment is lethal to all cells. These recent authors have each reviewed some of the extensive work reported in the last forty years, which shows that of both normal and neoplastic tissues in many species, some will and some will not survive exposure to extreme cold.

In view of this great variation in response to cold shown by different tissues, it was felt that an investigation into the effects of freezing and drying upon a tumour affecting a non-mammalian host would be of value; the Rous No. 1 fowl sarcoma was selected. Since it is a tumour from which a virus is easily separable, it was considered that the effects of cold and desiccation should be investigated upon the virus and the cells separately.

That the virus was able to survive freezing and drying has been established for some time (Knox, 1939*a*; Hoffstadt and Tripi, 1946; Dmochowski, 1948; Carr and Harris, 1951), and it has also been known to retain its tumour-producing activity after the application of cold by repeated freezing and thawing (Cramer and Foulds, 1930; Selbie and McIntosh, 1939).

However, the response of the cells of the Rous sarcoma to freezing and desiccation has not hitherto been ascertained. Many workers have assumed that

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Rous sarcoma cells could not survive desiccation (Rous, 1913 ; Rous and Murphy, 1913, 1914 ; Murphy and Landsteiner, 1925), but Nakahara (1926) was able to show that desiccated Rous material reconstituted with saline contained many cells apparently alive when subjected to vital staining, as well as many which showed movement in tissue culture. Similarly, repeated freezing and thawing has long been considered lethal to the cells (Rous and Murphy, 1914), although only Cramer and Foulds (1930) have offered any evidence for this. Both Simonin (1931) and Klinke (1940) have carried out tissue culture studies on the effect of a single application of cold on Rous cells ; neither was able to obtain evidence of cell growth after the treatment with the methods of culture which they used.

With regard to the problem of whether repeated freezing and thawing of Rous sarcoma tissue before extraction allows more virus to be liberated from the cells than is possible where no such treatment is applied, the evidence from previous work is scanty. Nakahara and Yaoi (1930) found that Rous sarcoma material which had been subjected to repeated freezing and thawing was less active when injected than similar untreated material, whilst Selbie and McIntosh (1939) were able to prepare from Rous tumour treated in this way filtrates with a greater tumour-producing activity than those made from untreated samples. Selbie and McIntosh (1939) considered that repeated freezing and thawing broke up the cells and allowed a greater liberation of virus than would otherwise occur, and the method has been used for obtaining good yields of Rous virus in various investigations (Knox, 1939*b* ; Pollard, 1939).

The work reported here consists of experiments upon the effects of temperatures just below — 70° C. and drying from the frozen state, on the viability of the cells and virus of the Rous No. 1 fowl sarcoma as judged by changes in their tumour-producing activity. An attempt has also been made to establish whether or not repeated freezing and thawing of Rous tumour affects the amount of virus which it will yield. A quantitative biological method has been used throughout, as suggested by Craigie (1949*b*).

MATERIALS AND METHODS.

Tumour.—The Rous No. 1 fowl sarcoma used was derived from that first introduced into the Bland-Sutton Institute in 1925, and maintained by passage since then.

Animals.—Pedigreed Brown Leghorn fowls from the Institute of Animal Genetics, Edinburgh, have been used in the experiments. They were between eight and nineteen weeks old when inoculated, apart from four exceptions which were slightly older, and belonged to three susceptible inbred lines developed at Edinburgh, or crosses derived from them. The lines and crosses used were Intensity, Breeding, Non-moult, Breeding hen × Intensity cock, and Intensity hen × Breeding cock, and have been described by Greenwood, Blyth and Carr (1948) ; both the age at which the birds were inoculated and the line or cross to which they belonged were determined by the exigencies of supply.

Preparation and treatment of tumour material.

For each experiment a bird with a large actively growing sarcoma of anything from 15 to 27 days' growth from the date of inoculation was selected as a source of tumour material and killed by wringing of the neck. The tumour was removed,

cut small with scissors, and disintegrated in a mechanical blender (" Ato-mix " of Measuring and Scientific Equipment, London); trapped air was removed from the disintegrated tumour by centrifugation. The method has been described in detail in a previous paper (Epstein and Cook, 1951). When prepared, the disintegrated tumour was loaded into a Record syringe for easy manipulation. Where disintegrated tumour was to be subjected to cold or desiccation, the treatment was applied as follows:

Freezing and storing.—Amounts of 1 c.c. of disintegrated tumour were loaded from a Record syringe with a long needle attached, into the bottom of ampoules having a stem of 6 mm. bore, and a bulb of 15·5 mm. bore (supplied by W. H. Edwards & Co., London). The ampoules were attached one after the other by a 2·5 cm. length of thick rubber tubing to the spindle of a 1/10 h.p. electric motor, suspending the ampoule vertically within a 2·4 cm. diameter shielding brass tube as has been described by Warner and Gostling (1950, Fig. 1). By starting the motor and setting the ampoule rapidly spinning on its longitudinal axis, a thin even film of disintegrated tumour was obtained on the inside of the bulb of the ampoule. With the ampoule spinning, a beaker of alcohol and carbon dioxide ice at a temperature just below — 70° C. was raised to surround the ampoule, thus very quickly freezing its contents in a thin shell. The ampoules were sealed when frozen and stored at −75° C. in an insulated carbon dioxide ice storage box; during the brief time required for sealing, thawing did not take place. When required, the ampoules were thawed by immersion in water at 22° C.

Drying from the frozen state.—The apparatus was essentially that employed by Craigie (1949*a*), and as used here has been described by Warner and Gostling (1950, Method I, Fig. 3). Before use, the apparatus was evacuated to give a pressure reading on the Pirani gauge of between 0·005 and 0 mm. Hg. Disintegrated tumour was shell frozen in ampoules as has just been described; when ready, the rubber tubes on the side arms of the manifold were clipped with Spencer Wells forceps, the glass rod stoppers were removed, and ampoules attached in their places. The forceps were then released, causing a rapid rise of pressure within the apparatus; this fell within five minutes to the original level, which was then maintained until the end of the period of drying, which lasted for 3 to 5 hours. At the end of this time the ampoules were removed, rapidly sealed, and stored at about 4° C. until required. When required, ampoules were opened, 1 c.c. of distilled water was added to each, and their contents were reconstituted by mixing with a sterile wooden stick.

Repeated freezing and thawing.—Disintegrated tumour was shell-frozen in ampoules as has been described, and was at once thawed. The process of freezing and thawing was rapidly repeated five times, after which the material was ready for use.

Preparations used for inoculation.

All samples of disintegrated tumour, both those left untreated and those subjected to freezing and storing, or freezing and drying, or repeated freezing and thawing, were made in each case into a virus suspension and a preparation of washed cells.

Virus suspension.—This consisted of 30 c.c. of a 10 per cent vol./vol. suspension of disintegrated tumour in physiological saline, from which the cells

were separated off by centrifuging in an angle centrifuge at 3500 r.p.m. for 15 minutes, decanting the supernatant fluid obtained, and recentrifuging it again in a horizontal centrifuge at 6000 r.p.m. for a further 15 minutes. The resulting clear supernatant fluid was decanted and used here as the virus suspension.

Washed cells.—This preparation consisted of the cellular material deposited during the angle centrifugation just described, washed by suspending in about 15 c.c. of saline and centrifuging in a horizontal centrifuge at 6000 r.p.m. for 15 minutes, resuspending the deposit in fresh saline, and performing the procedure either three (Experiment 1) or six (Experiment 4) times. The cellular deposit from the last washing was finally resuspended in a volume of saline equal to that of the original suspension (30 c.c.) from which it was derived and was used here as the washed cell preparation.

Experimental procedure.

Experiment 1.—Disintegrated tumour was prepared and was divided into various samples; one was left untreated, whilst each of the others was subjected to freezing and storing, or repeated freezing and thawing, or drying from the frozen state. All the samples of disintegrated tumour, both that left untreated and those subjected to one of the three forms of physical treatment, were then each made into a virus suspension and a preparation of washed cells; these were diluted in serial tenfold steps with saline ready for inoculation.

Experiments 2 *and* 3.—In each experiment a virus suspension was prepared from untreated disintegrated tumour in the manner described above; 1 c.c. was then added to 1 c.c. of each of two sera from fowl whose Rous tumours had regressed, and to 1 c.c. of pooled sera from 6 normal fowl. In addition, 1 c.c. amounts of the virus suspension were added to 1 c.c. volumes of dilutions of the sera made in serial tenfold steps with saline. The mixtures of virus suspension and neat or diluted serum were incubated in a water-bath at 37° C. for 30 minutes, and samples of each were then taken for inoculation. All the sera used were preserved by the addition of 1 per cent merthiolate in such quantity as to give a final dilution of 1 in 15,000.

Experiment 4.—In its early part this was a repetition of Experiment 1, in which virus and cell preparations were made from both untreated and treated disintegrated tumour; before serially diluting the preparations for inoculation, however, each was divided into two samples. One sample of each preparation was mixed and incubated with an equal volume of saline and the other with an equal volume of serum (diluted 1 in 10 with saline). The serum used (E) was from a fowl whose Rous tumour had regressed and was one which had been shown to have strong anti-Rous virus neutralizing powers in Experiments 2 and 3. In that part of the experiment in which the virus and cell preparations were made from disintegrated tumour subjected to freezing and storing (Experiment 4c), samples of the preparations were mixed with an equal volume of pooled normal serum (diluted 1 in 10 with saline), as well as with saline and Neutralizing Serum E. All the mixtures were incubated in a water-bath at 37° C. for 30 minutes, after which, part of each mixture was set aside ready for inoculation and part used to make dilutions in serial tenfold steps with saline, portions of each dilution being taken for inoculation.

Inoculations.—Inoculations were made into the breast and thigh muscles of

fowl; each inoculum was 0·5 c.c. in volume, and four inoculations of each preparation or dilution of a preparation were made.

When inoculating preparations whose tumour-producing potencies were to be compared with one another, the injections of material from each preparation were made into separate groups of birds; the scheme for the distribution of the injections of the various dilutions of each preparation amongst the birds in a group was the same in every case. Any possible masking of tumours which would have arisen late from inocula of high dilution, owing to the early death of birds from tumours developing rapidly from inocula of low dilution, was therefore spread equally in each group of birds.

Examination of fowl.—All the birds were examined every week, and all the tumours which developed, including those few which subsequently regressed, were recorded. Every bird which died or which was killed at the conclusion of an experiment was submitted to *post mortem* examination; histological investigations were carried out upon all material the nature of which could not be diagnosed macroscopically.

Duration of experiments.—The experiments were continued until all the tumour-bearing birds had died, or for a minimum period of six weeks, after which time the surviving birds were killed.

Calculation of results.

Inoculations were taken as having caused a tumour in all cases where tumours were palpated at the site of inoculation or where tumour material was found there at *post mortem*. From the number of tumours which arose from the inoculations of each dilution of a preparation, the dose of the preparation that would have produced tumours in 50 per cent of fowl was calculated in terms of c.c. of the disintegrated tumour from which the preparation was made. The method of calculation used was that of Reed and Muench (1938), and the figure obtained has been called by Warner and Gostling (1950) the TPD 50; it is shown in the tables by its negative logarithm, designated the "TPD 50 index." Comparison of this TPD 50 index of various preparations offers a comparison of their tumour-producing activities.

Strict aseptic technique was maintained throughout all the experimental procedures.

All the experimental procedures were timed. None was of more than 4 hours' duration up to the moment of completing the inoculations, thus standardizing the time in which gradual inactivation of the virus by oxidation (Pirie and Holmes, 1931) could take place.

RESULTS.

Table I shows that the tumour-producing activity of Rous virus suspensions made from disintegrated tumour which had been subjected to repeated freezing and thawing or freezing and drying was reduced as compared with those made from untreated material. Where the virus suspension was made from disintegrated tumour which had been frozen and stored, the tumour-producing activity was in one case greater (Experiment 1d) and in the other case less than that made from untreated material (Experiment 4d).

It will be seen from Table II that washed cells of the Rous sarcoma which had been exposed to any of the three physical treatments employed had less tumour-

producing activity than that of comparable untreated samples. The greatest reduction in activity was caused by freezing and drying (Experiments 1c and 4c).

TABLE I.—*The Effect of Repeated Freezing and Thawing, Freezing and Drying, and Freezing and Storing, on the Tumour-producing Activity of Preparations of the Rous Virus.*

Expt. No.	State of disintegrated tumour.	Preparation.	Dilutions of preparation and number of tumours arising from 4 inoculations of each					T.P.D. 50 index.
			10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
1a	Untreated	10% virus suspension	2+(2)	2+(1)	4	3	1	4·6
b	Frozen and thawed × 6		2+(1)	3	3	3	2	4·5
c	Frozen and dried		4	3	3	1	0	3·6
d	Frozen and stored 13 days		3	4	4	4	3	>5·3
4a	Untreated	5% virus suspension	3+(1)	3	4	3	3	5·6
b	Frozen and thawed × 6		3	3	2	2	1	3·8
c	Frozen and dried		3	3	4	2	0	4·2
d	Frozen and stored 28 days		2+(2)	3	4	1	0	4·1

Tumours which regressed shown in brackets.

TABLE II.—*The Effect of Repeated Freezing and Thawing, Freezing and Drying, and Freezing and Storing, on the Tumour-producing Activity of Preparations of Rous Sarcoma Cells.*

Expt. No.	State of disintegrated tumour.	Preparation.	Dilutions of preparation and number of tumours arising from 4 inoculations of each					T.P.D. 50 index.
			10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
1a	Untreated	10% washed (× 3) cells	3+(1)	3+(1)	2	3	3	5
b	Frozen and thawed × 6		3+(1)	2+(1)	2	2	0	3·6
c	Frozen and dried		2+(1)	1	1	0	0	2
d	Frozen and stored 13 days		3+(1)	3+(1)	3	3	1	4·6
4a	Untreated	5% washed (× 6) cells	4	4	4	4	1	5·3
b	Frozen and thawed × 6		4	4	4	3	0	4·9
c	Frozen and dried		4	4	2	0	0	3·6
d	Frozen and stored 28 days		3+(1)	3+(1)	3	2	0	4·4

Tumours which regressed shown in brackets.

TABLE III.—*The Effect of Various Fowl Sera on the Tumour-producing Activity of Rous Virus Suspensions when Mixed and Incubated with them.*

Expt. No.	Serum mixed with the virus suspension.	Dilutions of serum added to mixture and number of tumours arising from 4 inoculations of each mixture.			
		10^{-0}	10^{-1}	10^{-2}	10^{-3}
2	Pooled normal	3	4	4	4
	D	1	1	4	4
	E	0	0	0	3+(1)
3	Pooled normal	4	4	4	4
	C	1	1	2	4
	E	0	0	1	3+(1)

Sera C, D and E were from fowl whose Rous tumours had regressed
Tumours which regressed shown in brackets.

Table III shows the effect of various fowl sera, both neat and diluted in serial tenfold steps with saline, on the tumour-producing activity of samples of two different 10 per cent virus suspensions after incubation with them. Serum E, from a fowl whose Rous sarcoma had regressed, showed strong neutralizing powers.

From Table IV it is evident that washed Rous cells from untreated disintegrated tumour were rendered only slightly less active in producing tumours when incu-

TABLE IV.—*The Effect of Neutralizing Serum on the Tumour-producing Activity of Preparations of Rous Sarcoma Cells Treated in Various Ways.*

Expt. No.	State of disintegrated tumour.	Washed cells incubated with—	Dilutions of preparation and number of tumours arising from 4 inoculations of each				
			10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
4a	Untreated	Saline	4	4	4	4	1
		Serum E	2+(2)	2+(1)	0	1	1
b	Frozen and thawed ×6	Saline	4	4	4	3	0
		Serum E	0	0	0	0	0
c	Frozen and dried	Saline	4	4	2	0	0
		Serum E	0	1	0	0	0
d	Frozen and stored	Saline	3+(1)	3+(1)	3	2	0
		Normal serum	4	4	4	0	0
		Serum E	0	0	0	0	0

Tumours which regressed shown in brackets.

bated with neutralizing serum E than when incubated with saline (Experiment 4a). On the other hand, in the case of washed cells which had been subjected to repeated freezing and thawing or freezing and storing (Experiments 4b and 4d), incubation with neutralizing serum, in contrast with incubation with saline or normal serum, totally deprived them of their tumour-producing activity. Where the washed tumour cells had been subjected to freezing and drying, incubation with neutralizing serum (Experiment 4c) deprived them of most of their tumour-producing activity. One single tumour arose from all the inoculations made, resulting from an inoculation of the 1 in 10 dilution of the mixture of serum and washed cells.

DISCUSSION.

A number of points concerning the methods used require consideration before proceeding to an assessment of the results which have been obtained.

Disintegrated tumour was subjected to the various physical treatments under investigation in an undiluted state so that any possible effects attributable to the presence of diluents were avoided.

The technique of freezing disintegrated tumour in a thin shell in ampoules of standard size, as has been described, ensured that cold was applied as nearly as possible in a similar manner to each sample under test, as well as uniformly throughout individual samples.

Throughout the work the virus suspensions have been considered as being largely if not wholly cell free. On three occasions 40 c.c. samples of virus suspensions were subjected to very vigorous centrifugation (9000 r.p.m. for 30

minutes in a horizontal centrifuge), and the small quantity of structureless deposit obtained failed to show any intact cells when examined fixed and stained. Also arguing against the presence of more than an occasional cell in the virus suspensions is the fact that antiserum was able to neutralize their tumour-producing activity completely (Table III) even when diluted 1 in 20, since it has long been known that such antisera, active against the Rous virus, are ineffective against the cells (Rous, 1913; Sittenfield, Johnson and Jobling, 1931*a*, 1931*b*; Gye, 1931); indeed confirmation of this fact has been obtained in the present work (Table IV, Experiment 4a). Finally, Experiments 1 and 4 show that the tumour-producing activity of the virus suspensions was of the same order as that of the washed cells obtained in preparing them (Tables I and II). At the very least the centrifugation employed must be allowed to have separated off the overwhelming majority of the cells, and the great tumour-producing activity remaining with the virus suspensions cannot be attributed to occasional cells left in them.

Accepting therefore that the virus suspensions were almost entirely if not wholly cell-free, the ability of the Rous virus to retain some of its tumour-producing activity after exposure to cold has been demonstrated, thus confirming the work of Cramer and Foulds (1930) and Selbie and McIntosh (1939), who were able to prepare active filtrates from Rous tumour material subjected to repeated freezing and thawing. Confirmation has also been obtained of the ability of the virus to survive freezing and drying, which has been demonstrated by a number of previous investigators (Knox, 1939*a*; Hoffstadt and Tripi, 1946; Dmochowski, 1948; Carr and Harris, 1951). As in the work of Knox (1939*a*), so here the activity of virus preparations obtained from frozen and dried tumour material was considerably less than those made from untreated material (Table I, Experiments 1c and 4c). The contention, however, that preparations of Rous virus made from repeatedly frozen and thawed sarcoma had a greater tumour-producing activity than those made from untreated material on account of the treatment breaking up the tissue fragments and liberating the virus (Selbie and McIntosh, 1939) has not been borne out. The tumour-producing activity of virus suspensions made from repeatedly frozen and thawed samples of disintegrated tumour was considerably less than that of the suspensions made from comparable untreated samples (Table I, Experiments 1b and 4b).

With regard to the cells of the Rous sarcoma, the results of the present work show that cold or desiccation applied in any of the three ways under investigation reduced their tumour-producing activity as compared to that of similar untreated preparations (Table II). That the cell preparations were still active after the treatments could have been due either to the cells having survived in a viable state or to the presence of virus remaining attached to the remnants of the cells which the treatments had destroyed. It was considered that if the latter had been the case, and the tumour-producing activity of the treated cell preparations had been due to virus attached to dead cells, such virus would have been liable to inactivation if exposed to neutralizing serum. This supposition follows from the work of Rous, McMaster and Hudack (1935) with vaccinia and the virus of the Shope fibroma, which showed that neutralizing serum was only able to act upon virus unprotected by living cells. Experiments 2 and 3 (Table III) were performed to select a suitable anti-Rous virus neutralizing serum.

Now, washed cells which had been subjected to repeated freezing and thawing

or freezing and storing were totally deprived of their tumour-producing activity when exposed to the action of neutralizing serum (Table IV, Experiments 4b and 4d). Thus, in view of the work of Rous, McMaster and Hudack (1935), all the activity of the cell preparations treated in either of these two ways must have been due to virus attached to the remains of cells which the treatments had destroyed or liberated from such dead cells. The failure of the neutralizing serum to abolish the tumour-producing activity of washed cells which had been left untreated (Table IV, Experiment 4a) is considered to be evidence of the presence of live cells in the preparation.

The slight reduction in the tumour-producing activity of the untreated washed cells which was brought about by incubation with neutralizing serum is considered to be due to the action of the serum on free virus liberated from or attached to cells damaged during the disintegration of the tumour material.

The washed tumour cells subjected to freezing and drying were deprived of most of their tumour-producing activity after incubation with neutralizing serum (Table IV, Experiment 4c); out of the inoculations made with all the various dilutions of the frozen and dried washed cells, only one gave rise to a tumour. This was from one of the inocula of the 10^{-1} dilution of the mixture of cells and antiserum. Now, it has long been known that viruses can be recovered from neutral mixtures with antiserum simply by dilution of the mixture with saline; such reactivation has been demonstrated with vaccinia (Andrewes, 1928) and fowl plague (Todd, 1928), and although this "dilution phenomenon" is harder to obtain in the case of the Rous virus, Andrewes (1932) was able to show it conclusively. The single tumour resulting from the frozen and dried cell preparation which had been mixed with neutralizing serum (Table IV, Experiment 4c) followed the inoculation of a dilution of the mixture 1 in 10 with saline; it is considered to have been the result of a reactivation of virus in the neutral mixture by the process of dilution. This explanation of the origin of the tumour is favoured rather than one assuming the presence of cells in the inoculum which had survived freezing and drying, since simple freezing of the kind which preceded the drying was found in the present experiments to be enough to destroy Rous cells (Table IV, Experiment 4d).

The interpretation of the results of the present experiments which has just been put forward, namely that cold and desiccation destroy the cells of the Rous sarcoma, gains some support from the findings of other workers.

Thus, using Rous cells which had been exposed to a single application of cold, neither Simonin (1931) nor Klinke (1940) were able to obtain growth in tissue culture; though not conclusive, this was at least suggestive of cell death.

With regard to repeated freezing and thawing, Cramer and Foulds (1930) reported that where this treatment was applied to a slow-growing Rous tumour from which no virus could be extracted, all tumour-producing activity was abolished; this afforded the first experimental evidence for the belief, put forward long before by Rous and Murphy (1914), that such treatment destroyed the cells. It is of interest to note that the mechanism of the experiment performed by Cramer and Foulds (1930) was similar to that operating here, where repeatedly frozen and thawed material was exposed to neutralizing serum to eliminate the effects of free virus and establish whether or not viable cells were present. That the slow-growing tumour used by Cramer and Foulds (1930) failed to yield an active virus on extraction may be explained by assuming that it contained

inhibitor substance; from the work of Carr (1944) inhibitor substance is now recognized as being the same as serum antibody. This antibody played the same rôle in neutralizing the virus liberated from the frozen and thawed slow-growing tumour cells inoculated by Cramer and Foulds (1930) as that added to similarly treated material in the present experiments; in both cases the inocula were inactive, indicating that the cells had been killed.

Many workers have held the view, without offering any evidence in support, that drying destroys the cells of the Rous sarcoma (Rous, 1913; Rous and Murphy, 1913, 1914; Murphy and Landsteiner, 1925). The only investigations into this question (Nakahara, 1926) suggested in fact the opposite; Nakahara (1926) found that reconstituted desiccated Rous material showed cell migration, but not growth, in tissue culture. In relating this to the findings reported here it must be remembered that the drying process used was not preceded by freezing as in the present work, nor was it of comparable efficiency.

CONCLUSIONS.

The work reported here has approached the problem of the effects of cold and desiccation on the tumour-producing activity of Rous sarcoma material by attempting to keep distinct the action of these treatments on the virus and on the cells. It has been confirmed that the virus can retain its tumour-producing activity after freezing and storing, repeated freezing and thawing and drying from the frozen state as applied in this work. It has further been shown that this tumour-producing activity is less in the case of virus preparations made from repeatedly frozen and thawed or frozen and dried sarcoma material than that of preparations made from similar untreated material. No support has therefore been found for the contention that repeated freezing and thawing of Rous sarcoma tissue prior to extraction causes the liberation of more virus from the cells than takes place when no treatment is applied.

Freezing and storing, repeated freezing and thawing, and drying from the frozen state as practised here have all been shown to kill the cells of the Rous sarcoma.

SUMMARY.

Experiments are described, which were performed to establish the effects of cold and desiccation on the tumour-producing activity of both the cells and the virus of the Rous No. 1 fowl sarcoma. Susceptible inbred Brown Leghorn fowl have been used.

The method used to disintegrate Rous sarcoma tissue mechanically, and the technique of treating the disintegrated tumour material by freezing to below — 70° C., by repeated freezing and thawing, and by drying from the frozen state, are set forth.

A preparation of virus and a preparation of washed cells was made from all samples of disintegrated tumour, for inoculation into fowl; the methods are described.

A quantitative method has been used in which the dose of the preparations inoculated that would have caused tumours in 50 per cent of fowl was calculated in terms of c.c. of the disintegrated tumour from which the preparation was made. In this way the tumour-producing activity of different preparations has been compared.

The results show that the methods of freezing and storing, repeated freezing and thawing, and drying from the frozen state which were used here did not abolish the tumour-producing activity of the Rous virus. Repeated freezing and thawing when used before preparing virus suspensions from tumour material did not allow a greater liberation of virus than would otherwise have occurred.

Preparations of washed cells which had been subjected to cold and drying or had been left untreated were incubated with neutralizing serum to eliminate the tumour-producing activity of the free virus which they contained and allow the condition of the cells, as judged by their ability to cause tumours, to be investigated. The results obtained are considered to show that the cells of the Rous sarcoma were destroyed by freezing and storing, repeated freezing and thawing, and drying from the frozen state as applied in this work.

These results are discussed in detail.

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REFERENCES.

ANDREWES, C. H.—(1928) *J. Path. Bact.*, **31**, 671.—(1932) *Ibid.*, **35**, 243.
CARR, J. G.—(1944) *Brit. J. exp. Path.*, **25**, 56.
Idem AND HARRIS, R. J. C.—(1951) *Brit. J. Cancer*, **5**, 95.
CRAIGIE, J.—(1949*a*) *Ibid.*, **3**, 250.—(1949*b*) *Brit. med. J.*, ii, 1485.
CRAMER, W., AND FOULDS, L.—(1930) *Sci. Rep. Imperial Cancer Res. Fd.*, **9**, 33.
DMOCHOWSKI, L.—(1948) *J. nat. Cancer Inst.*, **9**, 73.
EPSTEIN, M. A., AND COOK, H. F.—(1951) *Brit. J. Cancer*, **5**, 244.
GREENWOOD, A. W., BLYTH, J. S. S., AND CARR, J. G.—(1948) *Ibid.*, **2**, 135.
GYE, W. E.—(1931) *Brit. J. exp. Path.*, **12**, 93.
Idem, BEGG, A. M., MANN, IDA, AND CRAIGIE, J.—(1949) *Brit. J. Cancer*, **3**, 259.
HIRSCHBERG, E., AND RUSCH, H. P.—(1950) *Cancer Res.*, **10**, 335.
HOFFSTADT, RACHEL, AND TRIPI, HELEN B.—(1946) *J. infect. Dis.*, **78**, 183.
KLINKE, J.—(1940) *Klin. Wschr.*, **19**, 585.
KNOX, R.—(1939*a*) *J. Path. Bact.*, **49**, 469.—(1939*b*) *Brit. J. exp. Path.*, **20**, 391.
MANN, IDA.—(1949*a*) *Brit. J. Cancer*, **3**, 255.—(1949*b*) *Brit. med. J.*, ii, 251.—(1949*c*) *Ibid.*, ii, 253.
MURPHY, J. B., AND LANDSTEINER, K.—(1925) *J. exp. Med.*, **41**, 807.
NAKAHARA, W.—(1926) *Gann*, **20**, 13.
Idem AND YAOI, H.—(1930), *Science*, **72**, 277
PASSEY, R. D., AND DMOCHOWSKI, L.—(1950) *Brit. med. J.*, ii, 1129.
Iidem, LASNITZKI, ILSE, AND MILLARD, A.—(1950) *Ibid.*, ii, 1134.
PIRIE, ANTOINETTE, AND HOLMES, BARBARA E.—(1931) *Brit. J. exp. Path.*, **12**, 127.
POLLARD, A.—(1939) *Ibid.*, **20**, 429.
REED, L. G., AND MUENCH, H.—(1938) *Amer. J. Hyg.*, **27**, 493.
ROUS, P.—(1913) *J. exp. Med.*, **18**, 416.
Idem, MCMASTER, P. D., AND HUDACK, S. S.—(1935) *Ibid.*, **61**, 657.
Idem AND MURPHY, J. B.—(1913) *Ibid.*, **17**, 219.—(1914) *Ibid.*, **19**, 52.
SELBIE, F. R., AND MCINTOSH, J.—(1939) *Brit. J. exp. Path.*, **20**, 443.
SIMONIN, C.—(1931) *C. R. Soc. Biol., Paris*, **107**, 1029.
SITTENFIELD, M. J., JOHNSON, B. A., AND JOBLING, J. W.—(1931*a*) *Proc. Soc. exp. Biol., N.Y.*, **28**, 517.—(1931*b*) *Amer. J. Cancer*, **15**, 2275.
TODD, C.—(1928) *Brit. J. exp. Path.*, **9**, 244.
WARNER, P. T. J. C. P., AND GOSTLING, J. V. T.—(1950) *Brit. J. Cancer*, **4**, 380.

THE INDUCTION OF TUMOURS OF THE BLADDER EPITHELIUM IN MICE BY THE DIRECT APPLICATION OF A CARCINOGEN.

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THE need for devising a technique for testing the carcinogenic properties of chemical compounds by direct application to the bladder epithelium has been felt for some time. Yamazaki and Sato (1937) described a method of introduction of oily solutions of chemicals into the rabbit bladder by catheterization. The interpretation of the true nature of the one acceptable tumour described by these authors after the introduction of o-aminoazotoluol is complicated by the presence of lipoid in phagocytes in the subepithelial tissues. In an extensive series of experiments, Hughes (1949), by means of epithelial grafts wrapped around 20-methylcholanthrene crystals, obtained 14 transplantable carcinomas from 120 attempts. He experienced difficulty in preventing contact of the carcinogen with connective tissue and thus inducing sarcomas. By a similar method, using the same chemical, Pan and Gardner (1948) obtained cancerous changes in one implant of bladder epithelium.

The technique described here was designed so that a known carcinogen would come directly into contact with the bladder epithelium for a protracted period, whilst the organ was functioning under normal physiological conditions. It was also desired to reduce the incidence of sarcomas and to obtain a higher yield of carcinomas than that achieved by the above authors.

METHOD.

Female mice of the Kreyberg albino strain and of a dealer's stock of large white albinos were used. A suspension of 20-methylcholanthrene in paraffin wax (melting-point 56° C.) was made. From this rounded pellets of approximately

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EXPLANATION OF PLATE.

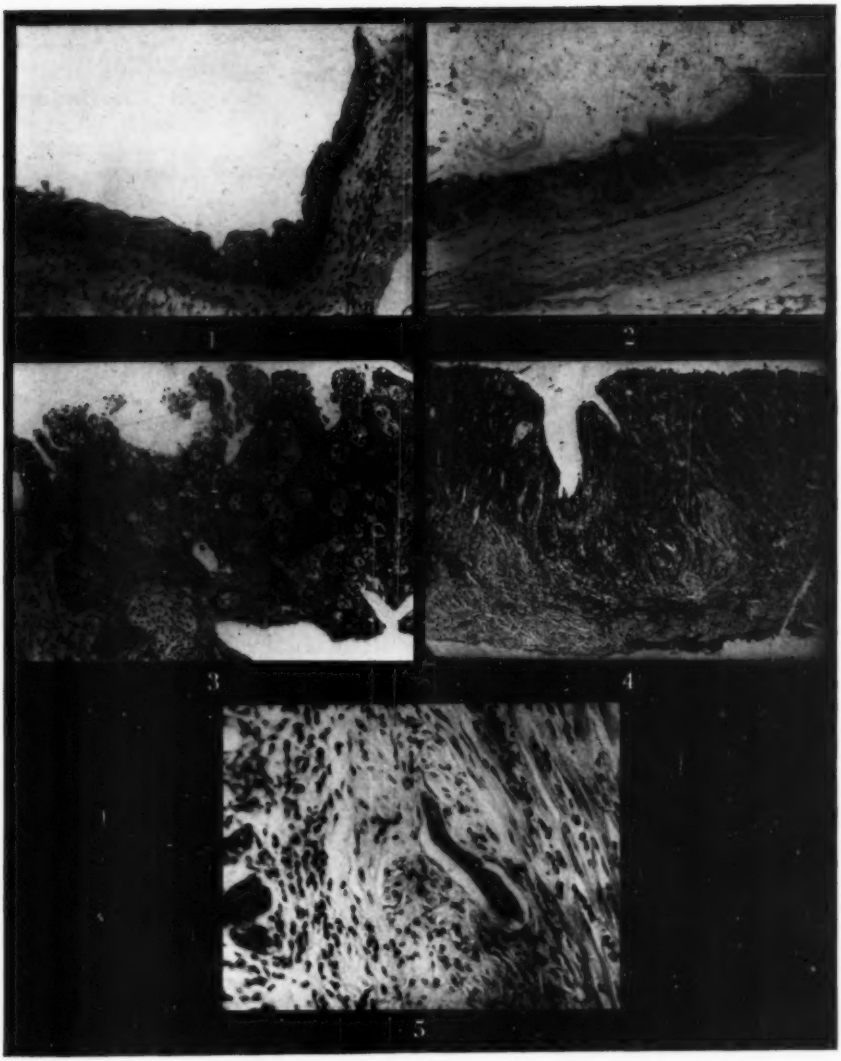
FIG. 1.—Base of bladder of female mouse implanted with pellet containing 2 per cent 20-methylcholanthrene for 42 weeks. The epithelium is markedly hyperplastic and hyperchromatic and rests directly upon the muscular coat. × 80.

FIG. 2.—Anterior wall of bladder, near base, of female mouse implanted with pellet containing 30 per cent 20-methylcholanthrene for 25 weeks. Hyperplastic epithelium at top right corner; the rest of the epithelium has undergone squamous metaplasia with keratinization and is showing subepithelial downgrowth. × 80.

FIG. 3.—Transitional cell papilloma situated at the dome in same mouse as in Fig. 2. The crypt at bottom right corner is due to folding. × 80.

FIG. 4.—Extensive squamous carcinoma situated on posterior wall in same mouse as in Fig. 2 and 3. The lining epithelium is intact and the tumour is invading the subepithelial tissues as far as the muscle coat. × 35.

FIG. 5.—Field adjacent to that seen in Fig. 4. On the left is the lower limit of subepithelial infiltration of the squamous cancer; in centre is an embolus of tumour cells in a lymphatic vessel deep in the muscular coat. × 165.



10 mg. weight were prepared by dropping the molten material from a pipette on to a chemically clean, sterile Petri dish. The pellets were moulded into shape by a warm scalpel. By this method an even distribution of the carcinogen was difficult to obtain, and so considerable variation in concentration may have occurred.

Under ether anaesthesia the bladder was exposed by a small incision through the skin and abdominal wall about 1 cm. above the urethral opening. The bladder was drawn out of the abdomen and supported on a small pad of gauze; an incision was then made through the dome of the bladder and the edges held apart by small metal retractors. The pellet was inserted by an assistant with forceps, and the incision in the bladder wall closed by a continuous suture of Gauge 0 silk thread using a Carrel's arterial curved needle. The suture passed through all the layers of the bladder wall, and the edges were drawn together so that the two epithelial surfaces were bound closely to one another; thus a continuous epithelial layer only and no muscle nor connective tissue was exposed to the direct action of the carcinogen in the pellet. The abdominal wall and skin were closed separately by sutures of cotton or thick silk.

The operative mortality was negligible, but a number of animals died within a few weeks of the operation due to the pellets blocking the urethra. Some of the animals showed signs of infection of the urogenital tract, confirmed by examining smears of the urine. This infection was palliated but not obviated by penicillin treatment.

The mice were killed by ether when they showed signs of failing health and the bladder sufficiently distended with Bouin's fluid, *per urethram*, to give an unwrinkled epithelium. The tissues were fixed in Bouin's fluid and paraffin sections stained with haematoxylin and eosin.

RESULTS.

Groups of 4 and 2 mice were implanted with pellets containing 2 per cent and 30 per cent suspensions of 20-methylcholanthrene respectively.

Mice treated with pellets containing 2 per cent of 20-methylcholanthrene.

On naked-eye examination after 21, 22 and 32 weeks of treatment there was slight irregular thickening of the bladder wall; after 42 weeks there was opacity of the bladder epithelium at the dome, i.e., at the site of suture, and at the base. Microscopically at 21 and 22 weeks hyperplasia of the epithelium was of slight degree, but at 32 and 42 weeks the change was well developed at the base and on the posterior wall of the bladder (Fig.1).

Mice treated with pellets containing 30 per cent of 20-methylcholanthrene.

On naked-eye examination after 25 weeks the bladder wall was generally thickened, with more marked diffuse epithelial thickening on the posterior wall of the dome, i.e., behind the suture line. At 34 weeks there was a small papilloma at the dome with epithelial thickening at the base. Microscopically, at 25 weeks a graded series of changes was seen in the bladder epithelium analogous to those described in dogs treated with 2-naphthylamine (Bonser, 1943). There was hyperplasia and squamous metaplasia with keratinization of the transitional epithelium of the bladder (Fig. 2). One well-developed

transitional cell papilloma (Fig. 3) was seen, and in addition, an extensive infiltrating squamous carcinoma reaching to the muscle but not actually invading it (Fig. 4). In a lymphatic vessel deep in the muscle was an embolus of tumour cells, leaving no doubt as to the malignant nature of the tumour (Fig. 5). At 34 weeks there was marked epithelial hyperplasia. The structure thought to be a papilloma on naked-eye examination proved microscopically to be a loose end of suture material covered with phosphatic deposit.

DISCUSSION.

As there was no precedent to guide us as to the optimum concentration of the carcinogen to be used in the pellets for this type of experiment it was decided to use 30 per cent and 2 per cent suspensions. The latter concentration had been found previously to be effective in inducing subcutaneous tumours, both sarcomas and breast adenocarcinomas (Bonser, personal communication). In the present experiment pellets containing 2 per cent of the carcinogen induced bladder epithelial hyperplasia but not tumours, whereas a pellet containing 30 per cent induced an infiltrating cancer. No doubt lower concentrations would be equally effective.

This technique has the advantage that any substance so applied to the bladder is subject only to the action of the urine before coming in contact with the bladder epithelium ; thus metabolic changes in chemical structure subsequent to feeding or injection are obviated. A reasonably high incidence of tumours should be obtained with an active substance without incidental induction of sarcomas.

It should now be possible to determine whether metabolites of bladder carcinogens such as 2-naphthylamine are the active agents in inducing bladder cancer. Economy in time, animals and material can be effected, and this is important when investigating the carcinogenicity of small quantities of metabolites isolated from urine. No high degree of skill is required to perform the operations described ; the time taken for one mouse has been about 20 minutes.

SUMMARY.

1. A technique is described for the local application of a carcinogen to the bladder epithelium over a prolonged period, whilst the organ is functioning under normal physiological conditions.

2. All of 4 mice treated with pellets containing 2 per cent of 20-methylcholanthrene showed patchy hyperplasia of the bladder epithelium.

3. Carcinoma of the bladder was induced in one of two mice treated with pellets containing 30 per cent of 20-methylcholanthrene. The other showed epithelial hyperplasia.

I wish to thank Dr. G. M. Bonser for her constant advice and invaluable assistance in interpreting the histological changes.

REFERENCES.

Bonser, G. M.—(1943) *J. Path. Bact.*, **55**, 1.
Hughes, B.—(1949) *J. Urol.*, **6**, 62.
Pan, S. C., and Gardner, W. U.—(1948) *Cancer Res.*, **8**, 613.
Yamazaki, J., and Sato, S.—(1937) *Jap. J. Derm. Urol.*, **42**, 332.

CARCINOGENESIS IN PARABIOTIC RATS.

TUMOURS OF THE OVARY INDUCED BY ACETYLAMINOFLUORENE IN INTACT FEMALES JOINED TO GONADECTOMIZED LITTER-MATES AND THE REACTION OF THEIR PITUITARIES TO ENDOGENOUS OESTROGENS.

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A PREVIOUS paper (Bielschowsky and Hall, 1951) described the tumours induced by 2-acetylaminofluorene (A.A.F.) in male rats joined in parabiosis to gonadectomized litter-mates. In the present communication an account will be given of the neoplastic changes obtained in female rats by the same experimental technique. The purpose of this investigation was to ascertain whether the endocrine imbalance created in the intact female rat joined to a castrated partner modified its response to the carcinogenic action of A.A.F.

METHODS.

The animals used belonged to a strain of Wistar rats with the exception of those of Group IV. These were females of a piebald strain of unknown origin obtained from Wallaceville Agricultural College. As in the males, a high rate of mortality occurred in pairs of parabiotic females during the second and third weeks after the rats had been joined in parabiosis. Additional losses occurred from the fourth month onwards in ever-increasing frequency. These were due mainly to the development of pyometra in the intact partner (Fig. 1). In an attempt to reduce the mortality due to this complication, the greater part of both horns of the uterus were surgically removed at the same time as the other partner was ovariectomized (Groups II and VI). The rats were 4 weeks old when these operations were performed and 7 days later the litter-mates were joined in parabiosis. The surgical technique used was the one recommended by Jacobsohn (1948). In most pairs both partners were females except in those forming Groups III and VII, where a castrated male took the place of the spayed female. They were joined at the age of 5 weeks, the male partner being castrated during this operation.

A.A.F. was administered by stomach-tube to the intact partner in doses of 4 mg. dissolved in 1 c.c. of peanut oil. A total of 10 to 25 doses was given, when possible in a schedule of 3 doses per week. Occasionally, this dosage had to be reduced to one or two administrations per week when the pair failed to show the expected gain in weight.

Twenty-six pairs of albinos (Groups I, II and III) and 3 pairs of piebald rats

(Group IV) compose the experimental groups treated with A.A.F. Only the pairs of Wistar rats which survived parabiosis for at least 16 weeks have been included in this account, since this was the earliest date when neoplastic changes appeared in intact partners of this strain. Groups V, VI and VII were not treated with A.A.F., and served as controls for the study of the effects of the hormonal imbalance created by joining a normal female to a gonadectomized litter-mate in parabiosis. The ten " single " female rats of Group VIII received 24 doses of A.A.F. by stomach-tube during the first 8 weeks of the experiment. These rats, like the majority of the parabiotic pairs, were 2 to 2½ months old when the administration of the carcinogen was commenced, and served as controls for the assessment of the carcinogenic activity of the amounts of A.A.F. given to the intact partners.

The rats of all groups received the ordinary laboratory diet as described in the previous communication (Bielschowsky and Hall, 1951). The parabiotic rats were sacrificed when declining health, in most instances due to the presence of large pyometra, made it advisable or when the presence of a tumour was suspected. Two of the " single " rats were killed when a palpable tumour was present, the remainder when the experiment was terminated at the 52nd week.

The material taken for histological study was fixed in Zenker, and the sections were stained with haematoxylin-eosin or according to Van Gieson. The pituitaries were fixed in sublimate formalin and stained by a modified Papanicolaou procedure. For the fat staining of the suprarenals the tissue was fixed in formol saline and stained according to Lillie's (1948) Oil Red O method.

RESULTS.

As in the case of the parabiotic males, tumours induced by A.A.F. were found only in the intact partner to whom the carcinogen was administered by stomach-tube, and there was no indication that the A.A.F. had affected the gonadectomized litter-mate. However, contrary to the results obtained in pairs of males not treated with A.A.F., long-standing parabiosis led to neoplastic growth in the intact female twin.

Tumours of the Ovary.

Tumours of the gonads were the neoplasms most readily induced in intact females treated with A.A.F. and joined in parabiosis to a gonadectomized litter-mate. The tumours originated in gonads which were grossly hyperplastic. The histological changes occurring in the ovaries of rats joined in parabiosis to gonadectomized litter-mates have been well described by Zeckwer (1944), therefore our findings will be mentioned only briefly. At the time when ovarian tumours appeared, normal corpora lutea were disappearing but corpus luteum cysts were still present. Large cystic follicles, already recognizable on naked-eye inspection, formed the bulk of these glands. Besides healthy follicles in all stages of development many athretic ones were also present. After 16 to 20 weeks of parabiosis such ovaries, even when free of large cysts and of neoplastic growth, had reached 4 to 5 times their normal weight. Microscopically, cystic follicles lined by granulosa or by a mixture of granulosa and lutein cells were a common occurrence at this stage, an indication of the abnormal stimulation to which the gonads were subjected. Apart from these changes ovarian cysts of varying size were seen ; they contained a clear or slightly turbid fluid, rich in

protein, which could be precipitated with diluted acetic acid. The wall of these cysts was smooth, and when an epithelial lining could be recognized the cells were low cuboid or flat without any indication of cellular activity. Such cysts occurred equally in intact partners treated with A.A.F. and in those which had not received the carcinogen. These cysts were considered not to be neoplasms. Another feature common to ovaries of rats of all groups were small haemorrhagic follicles. Large blood-filled cysts, however, were seen only in the ovaries of intact partners treated with A.A.F. They will be discussed together with the ovarian tumours. Table I lists the ovarian tumours obtained, including only

TABLE I.—*Tumours Induced by A.A.F. in Intact Female Partner of Parabiotic Rats.*

	Pair No.	Sex of partners.	Duration of experiment (weeks).	Number of doses of A.A.F.	Ovary.	Pituitary.
Group I	1	♀/spayed ♀	18	18	0	0
	2	Ditto	16	18	Gran. cell tum.	0
	3	„	19	18	0	0
	4	„	25	20	0	0
	5	„	20	18	0	0
	6	„	27	17	Gran. cell tum.	0
	7	„	38	17	0	0
Group II	1	♀/spayed ♀	19	10	0	0
	2	Ditto	41	14	0	Adenoma
	3	„	36	21	Carcinoma	„
	4	„	26	24	0	„
	5	„	19	21	Gran. cell tum.	0
	6*	„	43	21	„ „	Adenoma
	7	„	36	21	„ „	„
	8	„	19	21	„ „	0
	9	„	23	24	„ „	0
	10	„	20	25	0	0
	11	„	25	24	Gran. cell tum.	0
	12	„	28	24	Gran.-theca tum.	0
	13	„	40	24	0	Adenoma
Group III	1	♀/castr.♂	16	19	Gran. cell tum.	0
	2	Ditto	24	17	0	0
	3	„	35	20	0	Adenoma
	4	„	21	20	Gran. cell tum.	0
	5	„	31	20	Gran. cell tum. (left). Theca cell tum. (right)	0
	6	„	38	20	0	Adenoma
Group IV	1	♀/spayed ♀	13	24	Atypical proliferation of gran.	0
	2	Ditto	15	24	Gran. cell tum. (early)	0
	3	„	20	24	Gran. cell tum.	0

Groups I, II and III Wistar rats; Group IV Piebald rats.

* Multiple mammary tumours.

macroscopically recognizable neoplasms. Fourteen tumours of the ovary were found in Wistar rats (Groups I, II and III), an incidence of over 50 per cent, and 2 in Group IV consisting of three pairs of piebald rats. In one Wistar rat both ovaries showed neoplastic changes, in the others only one gonad was tumourous, the left and the right side being affected with equal frequency. In all but 3 of

the ovarian tumours granulosa cells were the prevailing cell type. Macroscopically they were of whitish colour, showing frequently areas of haemorrhagic discoloration. The whitish parts corresponded to solid, the reddish-brown ones to cystic blood-filled structures. The earliest neoplasms seen were generally round in shape and had a diameter of 3 to 4 mm. They were found in Wistar rats of Groups I to III after 16 to 20 weeks of parabiosis; in piebalds they occurred even earlier. The largest granulosa cell tumour which had destroyed completely the ovary in which it originated measured 27 × 16 × 25 mm. and weighed 3·88 g. It was the only neoplasm of this kind to produce a metastasis, the secondary being situated in the adhesions linking the primary to neighbouring organs.

The material at our disposal allows the study of the development of granulosa cell tumours from their early stages. Fig. 2 shows a large haemorrhagic cyst found in an ovary of Rat 1, Group IV, sacrificed at the 13th week of the experiment. This cyst had a thin wall formed by fibrous connective tissue which in most places was poor in cells. In some areas the capsule was thickened, and here signs of previous haemorrhage such as pigment-loaded macrophages were present. Into the cavity papillary projections protruded. They were of two kinds, some with a narrow and others with a broad base. Into the former, delicate strands of spindle-shaped cells entered, forming their core. Into the latter, capillaries penetrated surrounded by cells having ample eosinophilic cytoplasm, and a nucleous less rich in chromatin than the cells which covered and formed the bulk of the projections. These had a nucleus rich in chromatin and a scanty cytoplasm. They were typical granulosa cells, while the ones having ample cytoplasm resembled lutein cells. This was the earliest neopalstic lesion found in our material, and shows that atypical growths of this kind were not necessarily permanent ones. They may become firmly established and fill the whole of the cystic cavity or they may be destroyed by haemorrhages. We have seen frequently in ovaries obtained from rats treated with A.A.F. and killed after more prolonged parabiosis haemorrhagic cysts, where only small nests of granulosa cells remained embedded in the dense connective tissue of the capsule while the cavity contained only inspissated blood and tissue debris. Such chocolate cyst-like lesions were absent from the ovaries of the control pairs not treated with A.A.F. Another animal of the same group sacrificed 2 weeks later was found to have in the ovary a macroscopically visible nodule formed almost entirely by granulosa cells. It was of about the same size as the early granulosa cell tumuor depicted in Fig. 3, 4 and 5, which it resembled closely. This tumour was found in an A.A.F.-treated albino rat after 19 weeks of parabiosis (Group II, Rat 5). Fig. 3 shows a sharply limited spherical nodule situated in an ovary containing cystic follicles. It was surrounded by a thin fibrous capsule. Between the capsule and the neighbouring cystic follicles were large blood-filled sinuses. Into the nodule entered capillaries accompanied by cells which either resembled theca or lutein cells as illustrated in Fig. 4. Fig. 5 shows the appearance of the granulosa cells forming the bulk of the nodule, and the tendency towards foci of liquefaction leading to the formation of cystic spaces within the tumour. The remaining rat of Group IV (Rat 3) was killed at the 20th week of the experiment. Both its ovaries were grossly enlarged; the left weighing 600 g. is depicted in Fig. 6. A large whitish cyst forms the larger part of the gland. To the left of the cyst follows a white nodule having a few dark spots and the extreme left is occupied

by a dark blood-filled cyst. A section from the region of the white nodule shows two distinct solid structures, one nearly filling a cavity, while the other occupies only a fraction of a neighbouring cystic follicle (Fig. 7). Histologically both nodules were composed almost entirely of granulosa cells with an admixture of luteinized cells. As in the earlier tumours described above, small cystic spaces were present within the agglomerations of granulosa cells (Fig. 8). At first glance it seemed that some of these cavities were surrounded by tall cylindrical cells with a basically situated nucleus. Closer inspection revealed that in reality the layer nearest the cavity was formed by ghost cells. Their nucleus was hardly stained by haematoxylin, so that these elements by their close contact with adjacent healthy granulosa cells simulated cylindrical cells. A section of the largest granulosa cell tumour found in Rat 5, Group III, is shown in Fig. 9. This tumour had essentially the same structure as the smaller early neoplasm of this kind. It contained large cystic areas besides solid areas formed by granulosa cells arranged in folliculoid pattern or in solid agglomerations (Fig. 10).

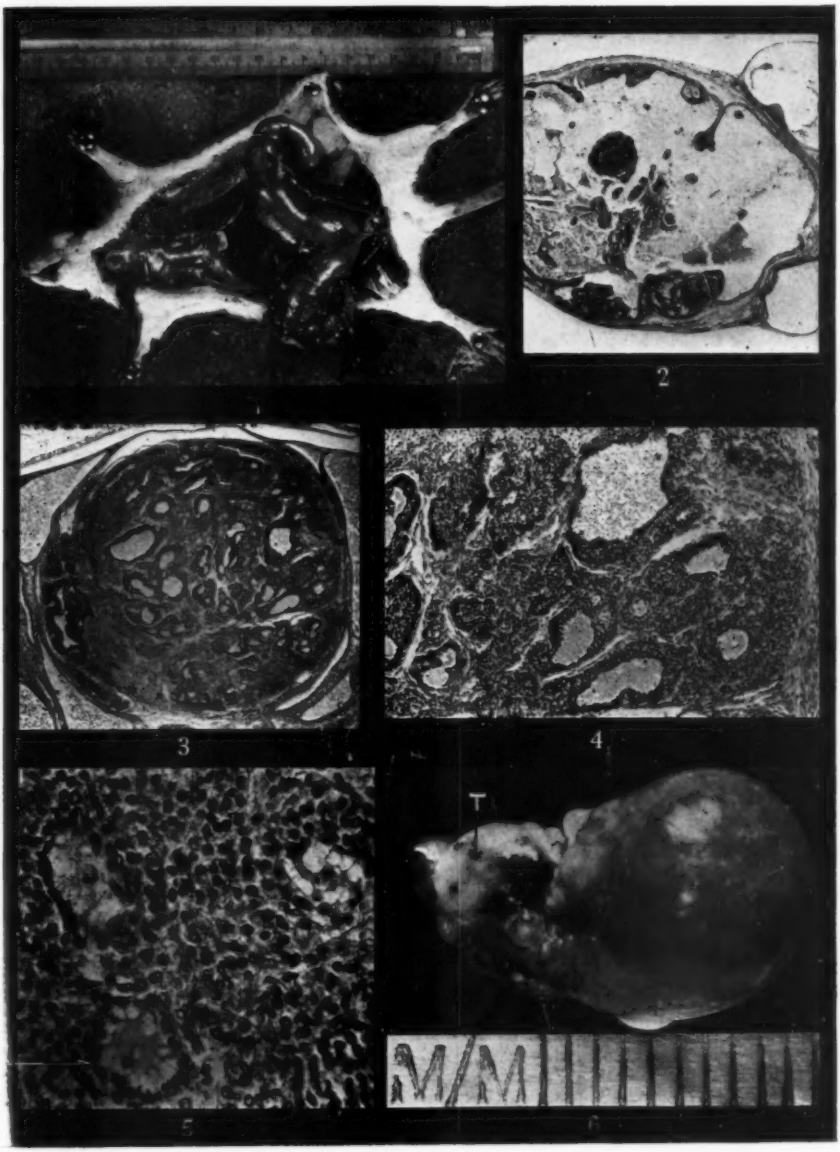
Only 3 tumours need to be described in greater detail. In Rat 12, Group II, the left ovary was greatly enlarged and its lateral half transformed into a hard tumour of whitish colour. Little ovarian tissue, apparently free of corpora lutea, was recognizable on naked-eye inspection. The organ weighed 286 mg., and was found to consist of areas of granulosa cells, nests of lutein cells and regions where theca-like cells predominated (Fig. 11). The tumorous right ovary of Rat 5, Group III, showed histologically an unusual picture. This ovary was also considerably enlarged (802 mg.) and fairly solid with only a few blood-filled cysts near its surface. Only a small rim of ovarian tissue remained; most of the specimen was obviously neoplastic. Histologically this tumour was composed mainly of spindle-shaped cells with scanty cytoplasm, and a nucleus which varied considerably from elongated dense to oval or spherical vesicular. Among them small groups of cells were present having ample cytoplasm which stained well with eosin. These elements had a larger vesicular nucleus with the chromatin displaced towards the nuclear membrane. They were arranged in tubular structures, the lumen being filled with colloid-like material. Both types had well-defined cell borders. The most prominent features of this tumour were glomerulus-like structures formed by the spindle cells (Fig. 12). Tufts of these elements grew into cystic spaces which rarely showed a typical lining. Rarely one gained the impression that cells resembling flat epithelium lined the cavity surrounding the tuft (Fig. 13). Only the largest of these structures contained a blood-vessel, which entered the tuft together with the spindle cells by a narrow pedicle. Finally in some areas of the tumour the appearance and arrangement of cells resembled closely that seen in the granulosa cell tumour of the other ovary. We have classified this neoplasm as a theca cell tumour because the granulosa contributed only little to its make-up.

Another unusual neoplasm was found in Rat 3, Group II. In the 36th week of the experiment the health of the intact partner suddenly declined and therefore the pair was sacrificed. In the region of the left ovary there was a large blackish cyst, on the posterior wall of which some ovarian tissue was recognizable. The anterior surface showed nodular whitish-grey tissue covering part of the cyst. Many adhesions connected the cyst with the omentum and the colon, from which it could not be severed. Multiple small reddish cysts of about 1 mm. in diameter were found in the omentum and other parts of the abdomen and the lungs were

riddled with nodules raised above the surface. These were of greyish colour or showed widespread haemorrhagic discoloration. Histological examination of the black cyst revealed that only in its periphery, i.e., near the fibrous capsule, cellular elements were present. These were of two kinds : tumour cells and cells of the granulation tissue which invaded from the periphery the inspissated blood and amorphous material filling the cyst. Two types of neoplastic cells could be distinguished : bizarre cells of unusually large size having one or, rarely, several nuclei with deeply stained prominent nucleoli. Their cytoplasm was ample and stained only weakly with eosin. Intermingled with these elements were nests of much smaller cells of epithelial character. These were polyhedral in shape and had well-marked cell borders, in contrast to the large elements which tended to form synyctia. The nuclei of the small cells were rich in chromatin and their cytoplasm was basophilic. The section reproduced in Fig. 14 was taken from the region where the tumour approached the colon. Here no invasion of the intestine is seen ; however, in other preparations it was found that the tumour cells had broken through the muscularis and invaded the submucosa. Widespread haemorrhages and lack of a stroma and of blood vessels were other characteristic features of this cancer. Fundamentally the same changes were seen in the lung. The larger the nodule the more prevailed haemorrhagic necrosis. Only the smallest deposits were free of haemorrhage, and consisted exclusively

EXPLANATION OF PLATES.

FIG. 1.—Intact parabiont with pyometra. × 0·3.
FIG. 2.—Haemorrhagic cyst with papillary ingrowths in hyperplastic ovary. × 12.
FIG. 3.—Early granulosa cell tumour. × 20.
FIG. 4.—Detail from Fig. 3 showing the capsule of the tumour, the blood supply, the arrangement of the granulosa cells and a nest of luteinized cells. × 65.
FIG. 5.—Detail from Fig. 3 showing the predominant cell type. × 330.
FIG. 6.—Hyperplastic ovary with clear and haemorrhagic cysts and early granulosa cell tumour. T indicates the area occupied by the tumour. (Pair 3, Group IV).
Fig. 7.—Section from tumour illustrated in Fig. 6 showing two distinct foci of tumour growth. × 13.
FIG. 8.—Detail from the larger nodule seen in Fig. 7. Granulosa cell tumour with admixture of lutein cells. × 90.
FIG. 9.—Section through the largest granulosa cell tumour observed. × 3.
FIG. 10.—Detail from tumour depicted in Fig. 9. × 65.
FIG. 11.—Area from a mixed granulosa-theca-lutein cell tumour. × 90.
FIG. 12.—Area from theca cell tumour showing " glomerulus-like " structures. × 110.
FIG. 13.—Another area of the theca cell tumour. × 330.
FIG. 14.—Area from anaplastic carcinoma showing the two types of tumour cells (L = large cell type, S = small cell type ; M = muscular coat of rectum). × 100.
FIG. 15.—Lung metastasis of tumour seen in Fig. 14. × 100.
FIG. 16.—Nest of atypical cells found in the ovary of intact partner (Pair 8, Group V). × 100.
FIG. 17.—Two small nodules found in the pituitary of the intact partner (Pair 13, Group II). × 65.
FIG. 18.—Detail from one of the nodules seen in Fig. 17 showing the transitions from fully granulated acidophils with large Golgi body to degranulated cells with similar Golgi apparatus. × 400.
FIG. 19.—Area from the other nodule seen in Fig. 17. Number of acidophils greatly reduced (appearing black in the microphotograph). Most cells have a large Golgi apparatus. × 240.
FIG. 20.—Section from fibroadenoma showing many secreting acini. × 60.
Fig. 21.—Atrophic uterus of the spayed partner of Pair 8, Group V. × 25.
FIG. 22.—Cystic hyperplastic breast gland of spayed partner of Pair 8, Group V. × 25.
FIG. 23.—Cortex of suprerenal of spayed partner of Pair 3, Group IV. Zona glomerulosa and outer fascicularis containing less lipoid than normally. (Oil Red O preparation.) × 60.
FIG. 24.—Cortex of suprarenal of a spayed rat. Glomerulosa loaded with fat droplets. (Oil Red O preparation.) × 60.



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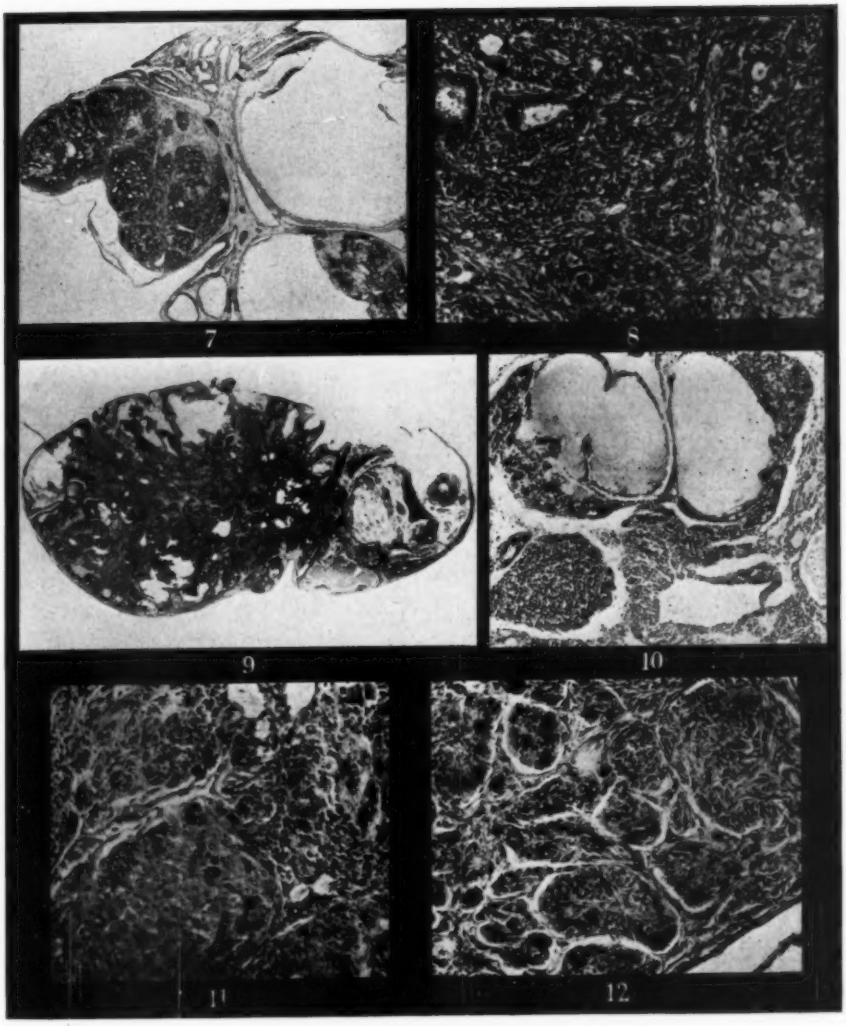

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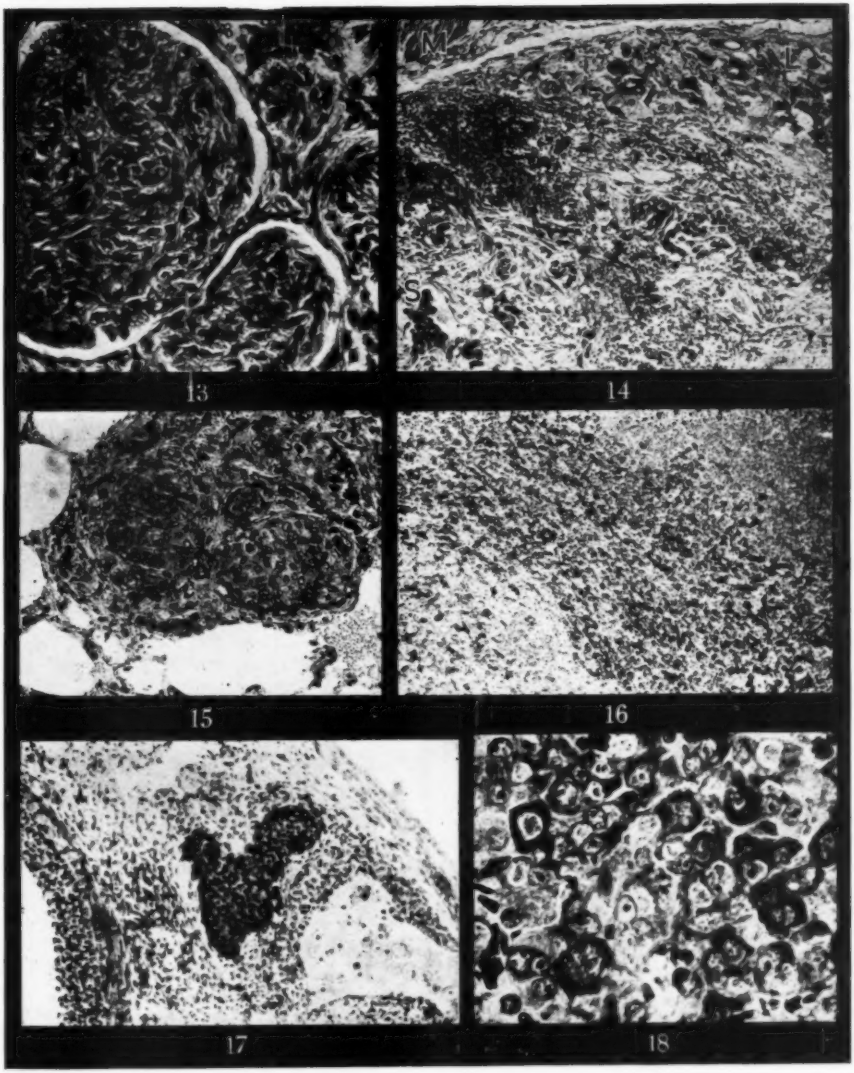

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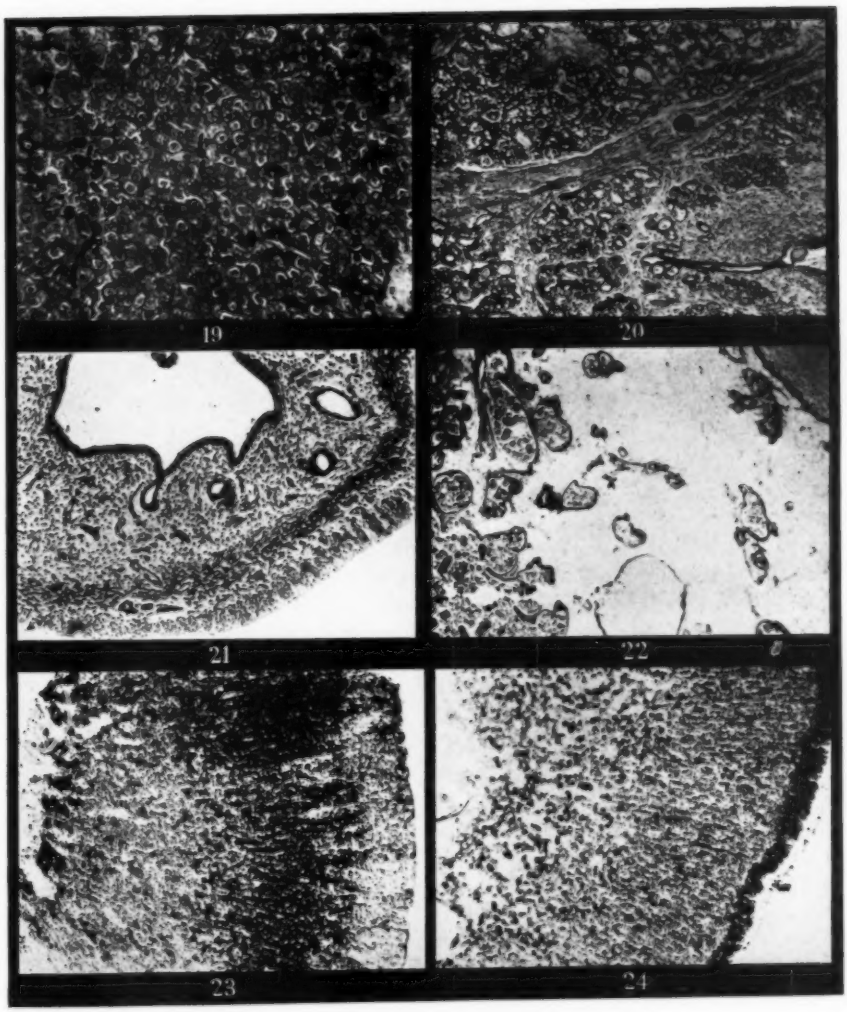

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of tumour cells as depicted in Fig. 15. Mitoses were as frequent in the primary tumour as in the secondaries. A thorough search did not reveal the presence of teratomatous structures in the primary, nor were we able to trace with absolute certainty the origin of the neoplasm to the ovary since the oviduct was no longer recognizable. Many features of this cancer resembled those of chorion-epithelioma.

As already mentioned, no tumours of macroscopic size were found in the ovaries of the intact parabionts not treated with A.A.F. (Groups V, VI and VII). The only lesion suspicious of atypical growth was found in one of the ovaries of an intact female which survived parabiosis for 42 weeks (Group V, Rat 8). An irregularly shaped but sharply limited nest of cells with basophilic cytoplasm and nuclei fairly rich in chromatin was seen situated near the surface of the ovary (Fig. 16). Serial sections revealed the presence of one to two mitoses per section, but an ovum was not found. We are unable to state with certainty that this lesion was of neoplastic nature.

As already mentioned, no ovarian tumours were found in the 10 "single" albino rats which received 24 doses of 4 mg. of A.A.F. by stomach-tube (Group VIII). Among many hundred female rats treated with this carcinogen we have found only one instance of a neoplastic lesion in the female gonads. This was discovered in a rat 17½ months of age when sacrificed. The rat had received 5 doses of A.A.F. by stomach-tube when 2 months old; then the animal was kept without further treatment for 1 year, and for the last 16 weeks of her life she received methyl-thiouracil in the drinking water. At the post-mortem the left ovary was found to be converted into a large cyst measuring 20 × 15 × 10 mm. On histological examination septa subdividing the cyst in several compartments and covered with granulosa cells were seen. Although macroscopically this cyst resembled the clear cysts which we have frequently found in the ovaries of parabiotic females and also in rats treated with stilboestrol, we have never observed in such structures proliferation of granulosa cells except in this one instance.

The morphological signs of the endocrine imbalance existing in the intact partner.

All signs of hyperoestrinism were present in the intact partner (Zeckwer, 1944). The vagina was lined by hyperplastic squamous keratinizing epithelium. In the few instances where the uterus was not heavily infected the epithelium lining the cavity was tall cylindrical and areas of squamous metaplasia were also present, the wall of the organ being thickened. In the infected uteri most of the epithelium was destroyed. Where rests remained it was generally squamous and keratinizing. The breast glands were grossly hyperplastic, and in most animals macroscopically visible cysts filled with a milky fluid were seen. Histologically such glands showed a pronounced cystic hyperplasia of the ducts and periductal fibrosis. The hyperplasia was not limited to the ducts, but alveolar growth had also occurred in many instances. The epithelial cells lining the alveoli showed marked secretory activity. The thymus was atrophic, and the suprarenals enlarged in size due to a hyperplasia of the cortex.

The pituitaries of the intact partners were invariably larger than those of the gonadectomized litter-mates, and already after 15 to 17 weeks of parabiosis had increased to an average of 8 mg. per 100 g. body weight. The largest observed (Rat 6, Group III, and Rat 8, Group V) reached the extraordinary weight of 74·8 and 139·3 mg. respectively. Such pituitaries produced signs of increased intra-

cranial pressure as ataxia and were an additional factor for the declining health in rats surviving parabiosis for more than 30 weeks. The pituitaries of the intact partners were mostly solid and of whitish colour and haemorrhagic areas were not conspicuous. While the majority of these glands showed a symmetrical enlargement of the anterior lobe, in females surviving for more than 25 weeks of the experiment the pituitaries assumed an ever-increasing irregular shape. At first small whitish or slightly yellowish nodules appeared which were single or multiple. Later on the normal shape of the gland became more and more obscured by irregular growths, which compressed but never invaded the brain. The larger the tumour the greater was the tendency for brownish discoloration. These lesions occurred in intact partners independently of whether A.A.F. was given or not (Tables I and II).

TABLE II.—*Adenomata of the Pituitary in Intact Female Partner of Parabiotic Rats not Treated with A.A.F.*

	Pair No.	Sex of partners.	Duration of experiment (weeks).	Pituitary.
Group V	1	♀/spayed ♀	17	0
	2	Ditto	20	Adenoma
	3	,,	22	0
	4	,,	32	0
	5	,,	30	Adenoma
	6	,,	18	0
	7	,,	26	0
	8	,,	42	Adenoma
	9*	,,	18	0
Group VI	1	♀/spayed ♀	23	Adenoma
	2	Ditto	32	,,
	3	,,	16	0
	4	,,	26	0
	5	,,	40	Adenoma
Group VII	1	♀/castr. ♂	25	0
	2	Ditto	21	Adenoma
	3	,,	28	,,

* All rats from Wistar strain except Pair 9, Group V (Piebald).

Microscopically the hyperplastic glands were free of basophils. The acidophils tended to be large ; they had a vesicular nucleus with a large nucleolus and a very prominent Golgi apparatus. The degree of degranulation varied considerably. The partially degranulated forms resembled closely a type of chromophobes characterized by an enlarged Golgi apparatus. These cells became increasingly numerous the longer the experiment lasted. The cells composing the early nodules of the pituitary differed little from those in the rest of the anterior lobe except for a reduction in the number of acidophils. Another feature of these foci of nodular hyperplasia was an increased number of mitoses. Fig. 17 shows two nodules separated by a slightly compressed strand of pituitary cells, among which acidophils are fairly numerous, and Fig. 18 an area of one of

these nodules photographed at a high magnification. In the latter all transitions from deeply granulated acidophils to apparently completely degranulated cells are recognizable. Common to many of these elements and independent of the degree of acidophilic granulation was the peculiar Golgi apparatus which in Papanicolaou preparations appeared as a ring-like structure, the negative Golgi image, surrounding some slightly brownish-stained granular material. Fig. 19, taken from the other nodule seen in Fig. 17, shows that the majority of the cells composing this structure were degranulated with only a few acidophils remaining. In the more advanced adenomata of the pituitary more atypical cells appeared but fundamentally they did not differ from the earlier lesions. In our material we have rarely seen adenomata completely free of acidophils. One or the other fully granulated and more frequently partially granulated acidophils were nearly always present. To conclude, we consider the nodular structures to be adenomata derived from cells of the acidophil series.

Tumours of other organs observed in intact partners and in " single " rats of Group VIII treated with A.A.F.

In the rats of Group VIII three adenocarcinomata of the breast and one squamous keratinizing epithelioma of the external auditory meatus were found (Table III). Two of the cancers appeared after an interval of 51 to 52 weeks, i.e., 9 weeks after the pair surviving longest had been sacrificed. Only one of the intact partners treated with A.A.F. developed tumours of the breast (Rat 6, Group II). In the 35th week of the experiment a mass was felt in the region of the fifth left mammary gland. It was surgically removed, and was found to be a well-encapsulated tumour measuring 20 × 12 × 9 mm. On cutting, milky fluid escaped under pressure. Histologically it was a fibroadenoma, in which the epithelial elements were much more numerous than is common in such tumours except when occurring in pregnant or lactating females. The breast gland in which the fibroadenoma originated showed the usual cystic hyperplasia typical for the mammary gland of intact partners and the tumour epithelium the same secretory activity as the glandular epithelium of the surrounding tissue (Fig. 20). Eight weeks later 2 more breast tumours appeared in this rat ; one was situated in the region of the 6th left breast gland and the other in the region of the 4th.

TABLE III.—*Tumours Induced by 24 Doses of 4 mg. of A.A.F. in " Single " Female Rats of the Wistar Strain (Group VIII).*

Rat No.	Duration of experiment (weeks).	Breast.
1	52	0
2	52	0
3	51	Adenocarcinoma
4	21	,,
5	52	0
6*	52	0
7	52	0
8	28	Adenocarcinoma
9	52	0
10	52	0

* Small carcinoma of external auditory meatus.

The former was macroscopically and histologically very similar to the neoplasm just described. The other was a small adenocarcinoma of the breast which showed far less secretory activity than the fibroadenoma which had developed simultaneously.

Benign cystic cholangiomata were present in practically all rats treated with A.A.F., but in the intact partners they were generally larger and more widespread than in the " single " animals, in which they were limited to a few cysts in lobus caudatus and left lobe. No malignant tumours of the liver were seen in any of these rats. The livers of the gonadectomized partners were always free of neoplastic lesions and were of normal size while those of the intact litter-mates were enlarged.

The spayed partner.

The pituitaries of the gonadectomized litter-mates showed always a marked increase in basophils, many of which were of the signet-ring type, and these glands were indistinguishable from those of " single " ovariectomized rats. The uterus and vagina were just as atrophic as in " single " castrates, but in some instances the breast glands were quite different from what we expected to see in a spayed animal. Instead of a few ducts lined by low cuboid epithelium many cystic ducts were present. The contrast between the atrophic uterus (Fig. 21) and the cystic dilated ducts of the mammary gland (Fig. 22) was especially striking in the spayed partner of Rat 8, Group V. We are unable to state why such changes occurred only in isolated instances.

Another feature in which the spayed parabiont differed from a " single " ovariectomized female was the cytology of the suprarenals. As depicted in Fig. 23 the zona glomerulosa and the outermost part of the fascicularis of the spayed partner showed a greatly diminished amount of lipoids, which was most pronounced in animals killed after 15 to 25 weeks of parabiosis. Fig. 24 is included for comparison ; it shows the distribution of lipoids in the cortex of the suprarenal of a " single " rat spayed 6 months previously. Here the zona glomerulosa was loaded with lipoids, and the outer fascicularis contained more droplets than towards the centre of the gland.

DISCUSSION.

Drips and Ford (1932), studying the effects of roentgen rays on the oestrous cycle and on the ovaries of the rat, were the first to observe neoplastic changes in the ovary as late sequels of irradiation. A few years later Furth and Butterworth (1936) described tumours of the ovary which occurred in old mice exposed to X-rays in their youth. A second experimental method of obtaining such neoplasms was discovered by Biskind and Biskind (1944), who obtained such tumours by transplanting ovaries into the spleen of gonadectomized rats. More recently Fels (1949) observed two ovarian tumours, one thecoma and one granulosa cell tumour, in rats subjected previously to a temporary ligature of the blood vessels entering the ovary. Finally, Moon, Simpson, Li and Evans (1950) described neoplastic lesions in the female gonads of rats treated with pure growth hormone for 1 to 1½ years. All the methods quoted induce an endocrine imbalance. X-ray radiation of the body as well as irradiation of the ovaries leads to partial or complete castration, as indicated by a more or less pronounced disturbance of the oestrous cycle, which is temporary or permanent, depending on the dosage given.

Lick, Kirschbaum and Mixer (1949), as well as Kaplan (1950), have shown conclusively that intact ovarian endocrine function inhibits the development of ovarian tumours in irradiated mice. In other words ovarian tumours develop only in animals in which the normal pituitary-ovarian relationship is disturbed for some time after the exposure to X-rays. Levine and Witschi (1933) found that a normal female rat joined in parabiosis to an animal whose ovaries had been exposed to X-rays went into continuous oestrus. The gonads of the intact partners were composed of cystic follicles—proof of increased secretion of follicle-stimulating hormone (F.S.H.) by the pituitary of the irradiated parabiont.

The liver of rodents inactivates ovarian steroids so efficiently that the secretions of a gonad grafted into the spleen do not enter the general circulation. In consequence the pituitaries of animals bearing such grafts are of the castrate type, and have an elevated gonadotropin content, as shown by Greep and Jones (1950). Fels (1949) discovered that a temporary ligature of the blood vessels supplying the ovaries led to an endocrine imbalance characterized by hyperoestrinism in the presence of castration changes in the pituitary. It seems therefore that one factor is common to at least 3 of the 4 methods quoted above; they induce an endocrine imbalance characterized by pituitaries secreting elevated amounts of gonadotropins. It is therefore not astonishing that the neoplastic changes induced under these experimental conditions are all of the same kind. Nearly all growths so obtained belong to the group of granulosa-theca-lutein cell tumours. Moon, Simpson, Li and Evans (1950) also regard the neoplastic changes observed in the ovaries of rats treated with growth hormone to be due to a pituitary imbalance. These authors mention a reduction of the acidophils and an increase of the chromophobes in the pituitaries of such rats, and noted that in 2 animals the histological picture of the anterior pituitary was of the castration type. It seems therefore that the basophils and especially the F.S.H. secreting cells can increase in animals treated with growth hormone. However, more information is required before one can assume that ovarian neoplasms obtained by treatment with growth hormone are due to the same mechanism as the other experimental tumours of the ovary, i.e., to an excess of gonadotropins.

In our case there can be no doubt that tumour development in the female gonad was the result of increased stimulation by gonadotropins and of the carcinogenic action of A.A.F. It is especially the follicle-stimulating hormone secreted in elevated amounts by the gonadectomized partner which determines the endocrine situation. Admittedly during the first weeks of parabiosis there is good evidence for the action of luteinizing hormone, as indicated by the presence of many healthy corpora lutea in the ovary of the intact litter-mate. Later, when the increased secretion of oestrogens from the stimulated ovary has suppressed all the gonadotrophic activity of the intact partner's pituitary and continuous oestrus has become established, the pituitary hormone dominating the picture is F.S.H. It is therefore not astonishing that granulosa cell tumours are so frequent in our material and that luteinization occurred to only a limited extent. We believe we have found enough evidence to postulate that these neoplasms originate from what Drips and Ford (1932) call hyperplastic follicular structures. Fig. 4, 5 and 7 of their paper resemble closely the early lesions observed by us, and which we have seen to progress to tumours of considerable size. It seems unfortunate that Drips and Ford (1932) compared the atypical structures observed by them in the irradiated ovaries of rats with carcinomatous ovarian cystadenomata of the

human. The undoubtedly decisive role of F.S.H. in the pathogenesis of the tumours described in this paper forces us to reject several of the hypotheses concerning the histogenesis of this class of ovarian neoplasms. There is general agreement that F.S.H. is responsible for the growth of follicles, but there is very little evidence that it is necessary for the formation of new follicles. The normal interplay between F.S.H. and luteinizing hormone (L.H.) in the ovarian cycle is greatly disturbed in the parabiotic female joined to a gonadectomized litter-mate. After 3 to 4 months of parabiosis the ripe follicle is not any more transformed into a corpus luteum nor does it involute, but is exposed to continued stimulation, which does not cease after the death of the ovum. This is in our opinion the decisive factor which leads to the development of the granulosa cell tumours. Contrary to what is seen in spleen grafted or irradiated ovaries of mice, in the rat there is not the slightest evidence that these tumours " arise from proliferation and ingrowth of germinal epithelium ", as assumed by Li and Gardner (1947). We have observed in some glands small tufts of cells growing out from the surface of the ovary, but we have never seen tubular ingrowths from " germinal epithelium ". Origin from embryonic nests seems a most unlikely hypothesis, since many workers obtained ovarian tumours in 50 to 70 per cent of their experimental animals (Kaplan, 1950 ; Furth and Sobel, 1948). Finally, origin from ovarian mesenchyme has to be considered. Undoubtedly the stroma cell of the ovary can differentiate into theca or granulosa cells, and therefore one cannot exclude this possibility. Still, we prefer origin from differentiated granulosa cells because of the morphology of the early lesions observed by us. They are frequently spherical, and surrounded by theca cells like normal follicles. In our opinion they arise from granulosa cells which survive the death of the ovum, the organization centre of the granulosa, and their point of origin are papillary ingrowths situated on the wall of cystic follicles. Furth and Butterworth (1936) also expressed the opinion that the granulosa cell tumours discovered by them in previously irradiated ovaries " may originate from remnants of disorganized follicles ".

That theca cells participated and in one instance became the main elements in the ovarian tumours obtained by us is in accordance with the hypothesis of follicular origin. If one tries to find an explanation for the " glomerulus-like " structures depicted in Fig. 12 and 13 one has not to fall back on the conception of disturbed embryogenesis. In preovulatory follicles invaginations of the theca accompanying tufts of capillaries are a common feature.

The chorion epithelioma-like tumour presented some diagnostic difficulties. We consider it to be an anaplastic carcinoma. As pointed out by Willis (1947), anaplastic carcinoma can simulate chorion epithelioma and therefore more than morphological similarity is required to establish the diagnosis, in the absence of pregnancy. The lack of teratomatous elements and the lack of signs of functional activity such as corpus luteum cysts in the unaffected right ovary are in favour of the diagnosis of anaplastic carcinoma. As far as the origin of this cancer is concerned we are inclined to assume that it arose in the left ovary, because we have observed more than once the presence of atypical cells of large size showing mitotic activity in the wall of haemorrhagic ovarian cysts. In addition it might be mentioned that Li, Gardner and Kaplan (1947) described small aggregates of giant cells in an experimental tumour of the ovary of a mouse. Also we have been unable to find references to tumours of the oviduct of rodents.

We have been impressed by certain similarities in the development of tumours of the thyroid induced by A.A.F. and prolonged treatment with a goitrogen and of the ovarian tumours which are the subject of this paper. In both cases a general hyperplasia of the glands is followed by a focal hyperplasia of a special kind which leads ultimately to malignant growth. If gonadotropines alone can induce ovarian neoplasms, as seems likely from the results of Biskind and Biskind (1944), one should expect them to appear also after long-continued parabiosis. So far we have been unable to maintain our pairs for more than 42 weeks, a period which seems too short to bring about this result without the aid of a carcinogen. To judge from the experience of Moon, Simpson, Li and Evans (1950) with growth hormone and of Griesbach, Kennedy and Purves (1945) with thyrotropic hormone, pituitary hormones appear to be slow acting carcinogenic agents. In both cases periods of more than a year were needed to obtain macroscopically recognizable tumours. However, tumour development in the thyroid was much more rapid, when A.A.F. was given simultaneously with or previously to the treatment with goitrogen.

Spontaneous ovarian tumours and especially granulosa cell tumours are exceedingly rare in rats (Iglesias, Sternberg and Segaloff, 1950), and therefore do not complicate the interpretation of the experiments reported in this paper. Finally an explanation has to be given why the incidence of granulosa cell tumours in Group I is lower than in Groups II and III. We believe that two factors are responsible ; lower dosage of A.A.F. and most probably also our surgical technique. Group I represents our first parabiotic pairs, in which we did not succeed as well as later on to obtain wide junctions, measuring 6 to 10 cm. at the time the animals were sacrificed.

The study of a number of organs of the intact partner showed clearly the effects of oestrogens secreted in excessive amounts by the stimulated ovaries. One of the sequels of this hyperoestrinism were the pituitary adenomata which occurred whether or not A.A.F. had been given. These neoplasms are indistinguishable from those obtained after long-continued treatment of rodents with large amounts of natural or synthetic oestrogens. Most authors describe them as chromophobe adenomata, and undoubtedly in advanced lesions only few granulated acidophilic cells are seen. We believe, however, that originally these benign neoplasms were acidophilic, just as in Zondek's case (1940). This author observed an adenomatous lesion composed of acidophils in the pituitary of a woman treated with very high doses of oestradiol.

Whereas the increased secretion of oestrogens by the stimulated ovaries led to neoplastic changes in the pituitary, no mammary tumours were observed in the control pairs, contrary to the findings of Zeckwer (1944), and only one of the intact parabionts treated with A.A.F. developed tumours of the breast gland. The material at our disposal does not allow the conclusion that the high level of oestrogen in the circulation of the intact partner inhibited the carcinogenic action of A.A.F. on the mammary gland ; but certainly this condition did not enhance it. On the other hand, the benign cystic cholangiomata of the liver were larger and far more widespread in the intact parabionts of Groups I to IV than in the "single" rats of Group VIII. This is in accordance with the findings of Stasney, Paschkis, Cantarow and Rothenberg (1947), who discovered that the administration of oestradiol " intensified the cytsic and hepatic lesions induced by A.A.F ".

SUMMARY.

(1) Benign and malignant tumours have been induced by A.A.F. in intact female rats joined in parabiosis to gonadectomized litter-mates. Ovarian tumours developed in about 50 per cent of these animals.

(2) A description of the histology of the ovarian neoplasms has been given and their histogenesis discussed. It has been found that most of them were of the granulosa cell type. F.S.H. secreted in excess by the pituitary of the gonadectomized litter-mate is regarded to be the factor essential for their development.

(3) The pituitary tumours observed in intact parabiotic females are considered to be due to the excess of oestrogens secreted by their stimulated ovaries. They occurred independently whether A.A.F. was given or not.

(4) The gonadectomized partners were always found to be free of neoplastic lesions, whether or not their litter-mates received A.A.F.

(5) The state of hyperoestrinism present in the intact parabiont did not enhance the carcinogenic action of A.A.F. on the mammary gland. When similar doses of A.A.F. were administered to "single" female rats two mammary cancers appeared during the first 40 weeks of the experiment, a period which corresponds to the maximum survival of our parabiotic pairs.

(6) Benign cystic cholangiomata were found in nearly all rats treated with A.A.F. In the intact parabionts they were more numerous and larger than in the "single" rats.

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REFERENCES.

Bielschowsky, F., and Hall, W. H.—(1951) *Brit. J. Cancer*, **5**, 106.
Biskind, M. S., and Biskind, G. T.—(1944) *Proc. Soc. exp. Biol., N.Y.*, **55**, 176.
Drips, D. G., and Ford, F. A.—(1932) *Surg. Gynec. Obstet.*, **55**, 596.
Fels, E.—(1949) *Obstet. Ginec. Lat.-Amer.*, No. 9, 439.
Furth, J., and Butterworth, J. S.—(1936) *Amer. J. Cancer*, **28**, 66.
Idem and Sobel, H.—(1948) *J. nat. Cancer Inst.*, **8**, 7.
Greep, R. O., and Jones, I. C.—(1950) *Recent Progress in Hormone Research*, **5**, 197.
Griesbach, W. E., Kennedy, T. H., and Purves, H. D.—(1945) *Brit. J. exp. Path.*, **26**, 18.
Iglesias, K., Sternberg, W. H., and Segaloff, A.—(1950) *Cancer Res.*, **10**, 668.
Jacobsohn, D.—(1948) *Acta physiol. scand.*, **17**, suppl., 57.
Kaplan, H. S.—(1950) *J. nat. Cancer Inst.*, **11**, 125.
Levine, W. T., and Witschi, E.—(1933) *Proc. Soc. exp. Biol., N.Y.*, **30**, 1152.
Li, M. H., and Gardner, W. U.—(1947) *Cancer Res.*, **7**, 549.
Iidem and Kaplan, H. S.—(1947) *J. nat. Cancer Inst.*, **8**, 91.
Lick, L., Kirschbaum, A., and Mixer, H.—(1949) *Cancer Res.*, **9**, 532.
Lillie, R. D.—(1948) 'Histopathologic Technic.' Philadelphia (Blakiston).
Moon, H. D., Simpson, M. E., Li, Ch. H., and Evans, H. M.—(1950) *Cancer Res.*, **10**, 549.
Stasney, J., Paschkis, K. E., Cantarow, A., and Rothenberg, M. S.—(1947) *Ibid.*, **7**, 356.
Willis, R. A.—(1948) 'Pathology of Tumours.' London (Butterworth).
Zeckwer, I. T.—(1944) *Arch. Path.*, **38**, 99.
Zondek, B.—(1940) *J. Amer. med. Ass.*, **114**, 1850.

PRECANCEROUS CHANGES INDUCED BY 20-METHYLCHOLANTHRENE IN MOUSE PROSTATES GROWN *IN VITRO*.

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MALIGNANCY was first induced in cells growing outside the organism by Earle (1943), who cultivated mouse fibroblasts *in vitro* in the presence of methylcholanthrene. Mouse fibroblasts, though exhibiting a certain architecture in culture, undergo unorganized growth in contrast to more differentiated tissues which, under suitable conditions *in vitro*, preserve the histological structure and sometimes the function of the organ from which they are derived. Since most tumours arise from highly differentiated organs, it seemed that a study of the direct effect of carcinogens on such tissues *in vitro* would be of great interest. The histogenesis of precancerous or malignant changes, if present, could be studied outside the organism, and the induction of precancerous changes or true malignancy would provide a convenient method to investigate, in controlled experiments, the influence of other factors such as hormones or co-carcinogens on this process.

Tumours of the prostate have been induced *in vivo* in mice and rats by implantation of carcinogens into the gland (Moore and Melchionna, 1937*a*, 1937*b*; Dunning, Curtis and Segaloff, 1946; Horning and Dmochowski, 1947).

Prostate glands of newly-born rats were found to grow satisfactorily *in vitro* (Price, 1949), and the cultures were maintained in good condition up to 25 days. This result encouraged the author to attempt to cultivate *in vitro* prostate glands of young mice. In preliminary experiments they were found to grow in culture for a similar period, and to continue their growth when grafted subcutaneously into the same strain of mice from which the explants were derived.

It was therefore decided to cultivate mouse prostate glands *in vitro* with the addition of 20-methylcholanthrene to the culture medium in order to find whether any changes could be induced by the carcinogen.

MATERIAL AND METHODS.

The prostates were obtained from C3H and Strong A mice approximately 6 weeks old (Fig. 1), and about 75 glands were used for the experiment. Usually the ventral prostates were explanted; they were grown by the watch-glass technique, which is eminently suitable for organized growth (Fell and Robinson, 1930). The methylcholanthrene was added to the culture medium in the following way: a solution of the agent in acetone was added slowly, with constant shaking, to human male serum. One drop of this mixture was added to the culture medium, which consisted of 4 drops of chick plasma (in some experiments 3 drops of chick and 1 drop of rat plasma) and 4 drops of chick embryo extract.

* Sir Halley Stewart Fellow.

The final concentration of the carcinogen in the medium was 2 gamma per c.c. in one series of experiments and 4 gamma per c.c. in another. The control cultures received serum to which acetone alone had been added. The medium was placed in a small watch-glass and allowed to clot; the watch-glass rested on a layer of sterile cotton-wool soaked with sterile distilled water inside a small Petri dish. At the beginning of the investigation the two lobes were grown together, but later this method was improved by explanting one lobe of each gland into medium containing methylcholanthrene while the other was kept as control. The explants were placed well flattened out on the surface of the plasma clot, where they became firmly anchored. After a day or two the growing explants liquefied part of the plasma clot, and while still firmly attached to the surface of the clot were surrounded by a pool of liquefied medium. The cultures were transferred to fresh medium every second or third day. After the ninth day no more methylcholanthrene was added, and the cultures were maintained in normal medium for the rest of the culture period.

The used glass-ware was decontaminated by keeping it in concentrated sulphuric acid at room temperature for at least one week. The cataract knives were brought into 5 changes of a solution of acetone and benzene in equal parts (Earle, 1943). The glands were fixed in 2 per cent acetic Zenker after the following periods of growth: 5 days, 10 days, 14 days and 21 days. Five to nine experimental and an equal number of control cultures were used for each point of observation. They were embedded in paraffin and serially sectioned; the sections were stained with Ehrlich's haematoxylin and eosin.

To assess mitosis the number of dividing cells was counted in every second section, i.e., in at least 20 sections of all single-lobe cultures. The increase in mitosis was then expressed graphically as the percentage of the control value, which was taken as 100.

RESULTS.

In the living explants two types of growth could be distinguished:

(1) organized growth consisting of the formation of new alveoli;

(2) unorganized growth consisting of a zone of fibroblasts surrounding the explant.

In general the outgrowth of fibroblasts was somewhat smaller and less dense in treated cultures, while the organized epithelium seemed to grow more satisfactorily and keep in better condition than that of the controls. At the end of the culture period it appeared completely healthy, with clear transparent edges, while in untreated cultures necrotic patches were sometimes seen at the growing edge; this observation *in vitro* was confirmed by the examination of the stained serial sections.

I. *Normal untreated cultures.*

Usually active growth takes place at the periphery of the explant, where new alveoli are being formed while the centre undergoes some degeneration.

As cultivation *in vitro* goes on the necrotic matter is resorbed and replaced by new, usually wide alveoli. In both peripheral and central alveoli the epithelium loses its glandular character; the epithelial folds characteristic for the glands *in vivo* disappear, the epithelium becomes low and secretion is rare.

Fig. 2 shows a typical control culture after 10 days' growth in which wide central alveoli and smaller peripheral ones can be distinguished. The alveoli are lined by one layer of low epithelial cells of regular size and shape in which mitosis is present though infrequent. (The rate of mitosis was found to vary slightly in different batches of cultures, but this difference seemed independent of the length of cultivation.) The stroma is dense and much increased as compared with that of the original gland and the treated cultures. Fig. 9 shows another control after a fortnight's growth with cystic dilatation of most alveoli and relatively dense stroma. Thus in general, the cultures resemble the prostate glands of castrated mice.

II. Cultures treated with 20-methylcholanthrene.

Cultures treated with methylcholanthrene show a similar type of growth at the beginning of the culture period : formation of new alveoli at the periphery and some central degeneration. The latter, however, is much less marked than in the controls. The epithelium retains its glandular character and remains actively secreting in the treated explants for a longer time, but the stroma is usually very poor and reduced in both cells and fibres.

Apart from this preservation of its glandular character other more striking changes of the alveolar epithelium can be observed in treated cultures. These consist briefly of a considerable increase in cell division, hyperplasia of the lining epithelium and squamous metaplasia. In some cases foci of anaplastic cells with abnormal mitotic figures and cells of irregular size and shape can be distinguished. Table I gives the total number of treated cultures fixed at the different

TABLE I.—*The Number of Treated Cultures Showing Hyperplasia and Squamous Metaplasia.*

Days during or after treatment.	Hyperplasia		Squamous metaplasia.	
	2γ.	4γ.	2γ.	4γ.
5	2/6*	2/5	0/6	1/5
10	6/7	5/5	0/7	1/5
14	8/9	7/7	1/9	4/7
21	5/6	6/6	5/6	6/6

* The right-hand figures give the total number of cultures fixed at each point of observation.

points of observation and the number of those showing hyperplasia and squamous metaplasia at those times.

Fig. 3 shows the increase of mitosis in treated cultures relative to the control value over the period of 21 days. Two distinct waves can be recognized ; the fall in mitosis follows closely the removal of the carcinogen.

After 5 days' growth mitosis is increased with both concentrations to 3 to 6 times the control value. With the higher concentration one culture out of five already shows some hyperplasia with squamous changes (Fig. 4).

After 10 days' cultivation, i.e., one day after the removal of the carcinogen, the proliferative changes are more marked. Nearly all treated explants show alveoli with hyperplasia of the lining epithelium, which now consists of several layers of densely packed cells (Fig. 5), which at this time are mostly glandular in character (Fig. 6). Frequently only one part of the alveolar wall is involved,

and elsewhere the epithelium is composed of a single layer of apparently healthy cells. One out of five treated cultures shows squamous metaplasia after the higher dose. Mitosis is still above that in the controls and has, in fact, risen after the higher concentration while it has dropped after the lower. Table II gives the number of mitotic cells counted in 20 sections of controls and cultures treated for 9 days with 2γ/c.c. of methylcholanthrene and fixed after 10 days' growth.

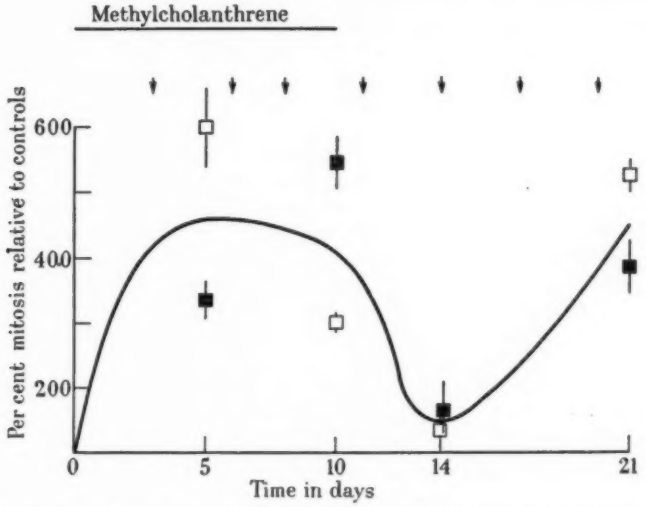


FIG. 3.—Increase in mitosis in prostate cultures treated for 9 days with 2γ and 4γ/c.c. of methylcholanthrene over a period of 21 days. The arrows indicate subcultivations.
□ = 2γ. ■ = 4γ.

TABLE II.—*The Number of Mitotic Cells in Controls and Cultures Treated with 2γ/c.c. of Methylcholanthrene Fixed after 10 days' Growth.*

Controls.	Experimental.	E/C per cent.
34	90	265
23	66	287
31	85	274
30	108	360
14	44	316
26	76	292
	Average	299%±14·1

In cultures fixed after a fortnight's growth *in vitro* and 5 days after withdrawal of the carcinogen, the hyperplastic changes are still more pronounced. Papilliform processes can be seen projecting into the lumen of many alveoli (Fig. 7), while in others the lumen is partially or completely occluded. Moreover the cells have become more irregular in shape and size (Fig. 8). After the higher concentrations

the epithelium has undergone squamous changes in over half of the treated cultures (Fig. 10). The number of dividing cells has fallen and nearly reached the control level.

At the same time changes of a more anaplastic type can be observed in one batch of cultures treated with 2γ of methylcholanthrene. Abnormal mitotic figures of great size showing polyploidy, aberration and breakage of chromosomes can be seen among the epithelial cells lining the alveoli (Fig. 18). At the same time single very large mononucleate or multinucleate cells can be distinguished (Fig. 16 and 17). Frequently foci consisting of anaplastic cells of irregular shape and widely different sizes, some of them degenerate and parakeratotic, make their appearance (Fig. 15).

After 21 days' growth, 11 days after removal of the carcinogen, most cultures show squamous changes of the hyperplastic epithelium (Fig. 10 and 11). Many alveoli are partly or completely filled with layers of basal, parakeratotic and keratinizing cells; often genuine prickle cells and cells with keratohyalin formation are present (Fig. 13). The basal cells usually form the peripheral layers, while the keratinizing elements occupy the centre of the alveolus, where they are often shed into the lumen (Fig. 12, 13). Where only part of the alveolar wall is involved, the remainder of the epithelium is still secreting, and often alveoli are found filled partly with squamous epithelium and partly with secretion (Fig. 13).

Mitotic figures, most of which are normal, are frequent among the basal cells and cell division shows another rise to 4 to 5 times the control value. The ratio of basal to differentiating cells varies slightly in different cultures. In some explants a high proportion of basal cells is associated with a high rate of mitosis (Fig. 12, 14); in others the ratio is reversed in favour of the differentiating cells and mitosis is less frequent (Fig. 13).

DISCUSSION.

The results described indicate that methylcholanthrene in the doses used *directly* promotes the rate of cell division and causes hyperplasia and squamous metaplasia of the alveolar epithelium. The first changes are seen as early as 5 days after the beginning of treatment; they become more pronounced as the treatment continues and persist after the removal of the carcinogen. Squamous metaplasia develops more fully, particularly after the smaller dose, after the end of treatment.

The increase in mitosis shows two distinct waves. It is noteworthy that the downward bend of the first wave coincides with the end of treatment. This may mean that the first peak is caused by a direct stimulating action of the carcinogen, while the second one is due to the increased growth-potential of the changed epithelium. A rise in cell division has been reported by Cooper and Reller (1942), who found a ten-fold increase in the mitotic rate in the skin of mouse ears painted with methylcholanthrene, and by Glucksmann (1945), who observed a rise in mitosis a few hours after a single painting with benzpyrene.

In contrast to the increase in mitosis and proliferation of the epithelial cells is the reduction of the stroma of the treated explants. The question arises whether this is an indirect effect secondary to the increased epithelial growth, or whether it is due to a direct inhibition of fibroblastic growth by the carcinogen.

Earle and Voegtlin (1938) and Earle (1943) have shown that the growth of

mouse and rat fibroblasts *in vitro* is much inhibited by a concentration of 1γ/c.c. of methylcholanthrene in the culture medium, and that increasing the dose produces severe cell degeneration. This and the smaller zone of fibroblasts observed around the treated cultures in the present experiments suggest that the carcinogen may, at least partly, be responsible for the poor stroma in the prostate cultures, and raises the question of whether this inhibitory effect is a contributory factor in the carcinogenesis of epithelial tumours produced by carcinogenic hydrocarbons *in vivo*. This difference of effect, however, does not seem to be due to a differential action of the carcinogen on epithelium and fibroblasts, but seems to be one of degree. Creech (1940) obtained mitotic stimulation in cultures of mouse fibroblasts treated with 0·015 γ/c.c. of methylcholanthrene, which indicates that the dose for stimulation of fibroblasts is roughly twenty times lower than that for epithelium if the concentrations of 2–4γ/c.c. used in the present experiments are taken as the growth stimulating dose for (prostatic) epithelium.

The effect of these two concentrations is qualitatively similar. After 4γ/c.c. however, the increase of mitosis is delayed, and does not reach the same high level as after the lower dose. This may mean that at 4γ/c.c. the inhibitory effect of the carcinogen already comes into play. Squamous metaplasia, on the other hand, appears sooner after the higher dose (Table I). This result suggests that promotion of cell division and interference with normal differentiation are not related, but independent processes.

EXPLANATION OF PLATES.

FIG. 1.—Section through a normal prostate gland *in vivo*. × 120.

FIG. 2.—Control culture after 10 days' growth. Note low epithelium in centre and dense stroma. × 120.

FIG. 4.—Culture grown with 4γ/c.c. of methylcholanthrene fixed after 5 days' growth. Note glandular type of epithelium and hyperplasia in two alveoli, one with squamous change. × 120.

FIG. 5.—Culture treated for 9 days with 2γ/c.c. of methylcholanthrene and fixed after 10 days' growth. Note hyperplastic epithelium, secretion and lack of stroma. × 120.

FIG. 6.—Alveolus with hyperplastic epithelium in a culture treated with 2γ/c.c. of methylcholanthrene for 9 days and fixed after 10 days' growth. × 400.

FIG. 7.—Hyperplastic epithelium projecting into alveolus in a culture treated with 2γ/c.c. of methylcholanthrene for 9 days and fixed after a fortnight's growth. × 400.

FIG. 8.—Occlusion of an alveolus in a similar culture. Note variation in cell size and shape. × 400.

FIG. 9.—Control culture after 14 days' growth showing wide alveoli with low epithelium and well-developed stroma. × 120.

FIG. 10.—Culture treated with 4γ/c.c. of methylcholanthrene for 9 days and fixed after a fortnight's growth showing partial occlusion of alveoli with squamous metaplasia. × 120.

FIG. 11.—Culture treated with 2γ/c.c. of methylcholanthrene for 9 days and fixed after 3 weeks' growth showing partial and complete occlusion of alveoli with squamous metaplasia. Note secretion. × 120.

FIG. 12.—Part of an alveolus with squamous metaplasia in a culture treated with 4γ/c.c. of methylcholanthrene for 9 days and fixed after 2 weeks' growth. Note high proportion of basal cells, and mitosis. × 400.

FIG. 13.—Squamous metaplasia in a culture treated with 2γ/c.c. of methylcholanthrene for 9 days and fixed after 3 weeks' growth. The number of basal cells is smaller and mitosis lower than in Fig. 11. Note keratohyalin granules and secretion. × 400.

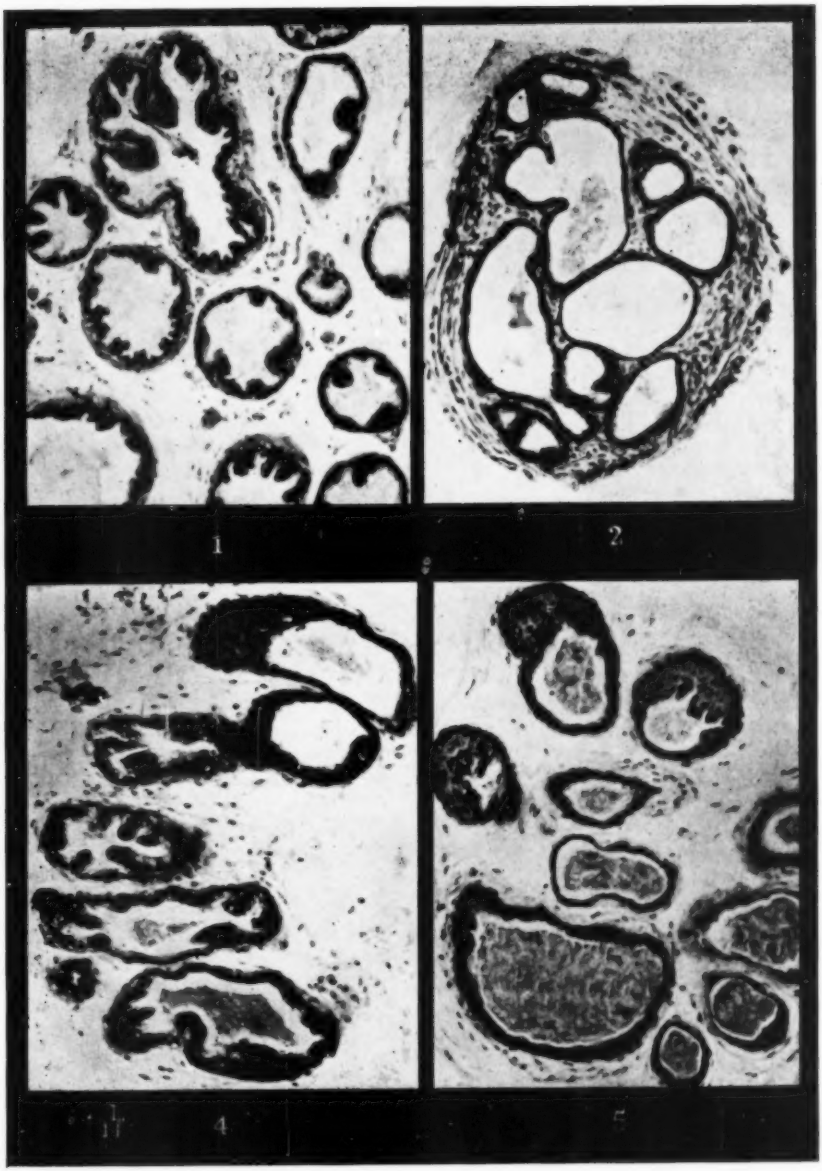
FIG. 14.—Epithelial strand growing away from alveolar edge in similar culture. High proportion of basal cells with high incidence of mitosis. × 400.

FIG. 15.—Anaplastic cells in a culture treated with 2γ/c.c. of methylcholanthrene and fixed after 2 weeks' growth. × 400.

FIG. 16.—Single large cell in similar culture. × 730.

FIG. 17.—Single large cells in alveolar lining among others of normal size. × 600.

FIG. 18.—Polyploid cell division in alveolar lining. Note cell of normal size for comparison. × 1300.



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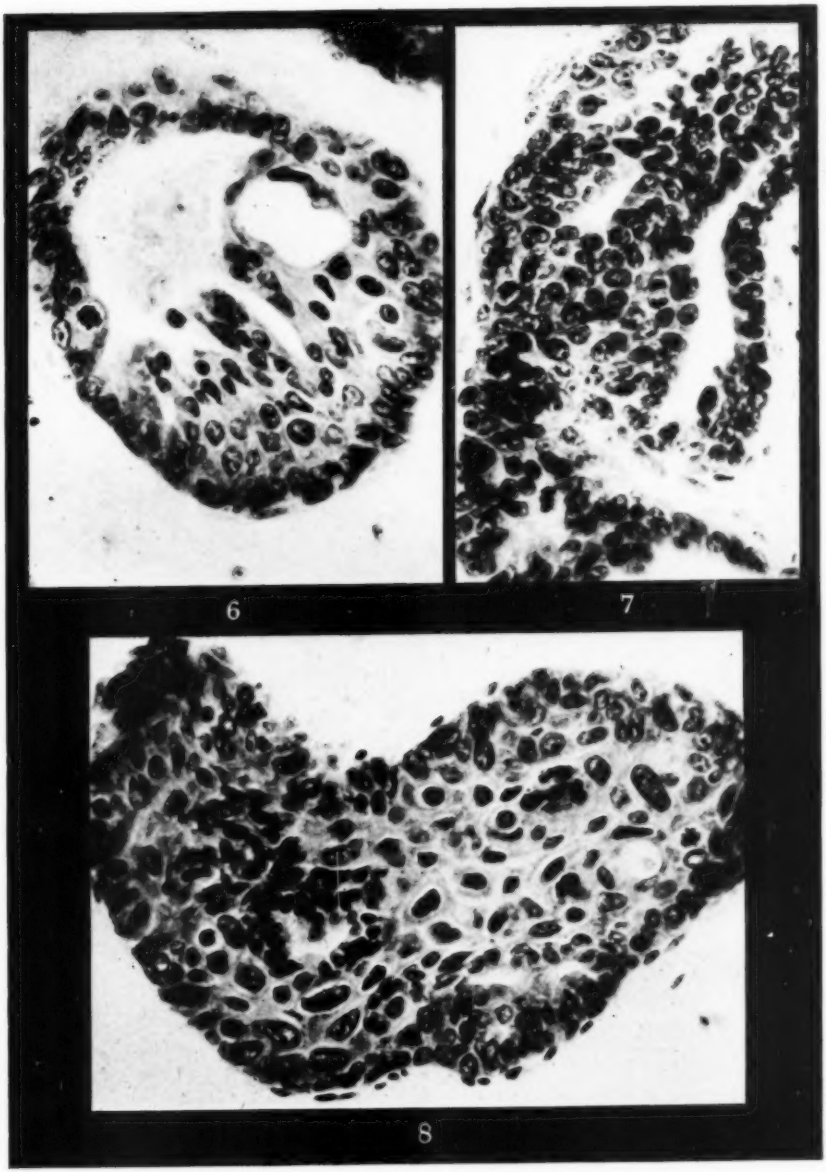

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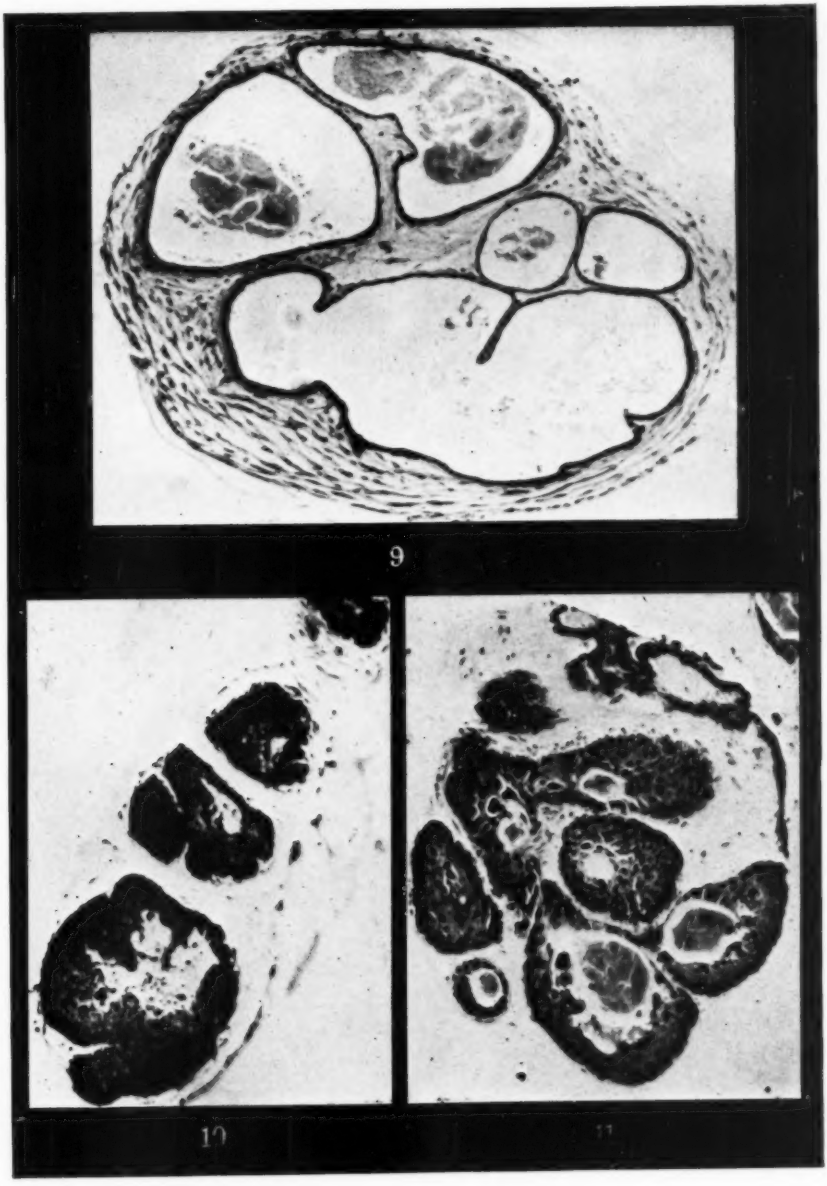

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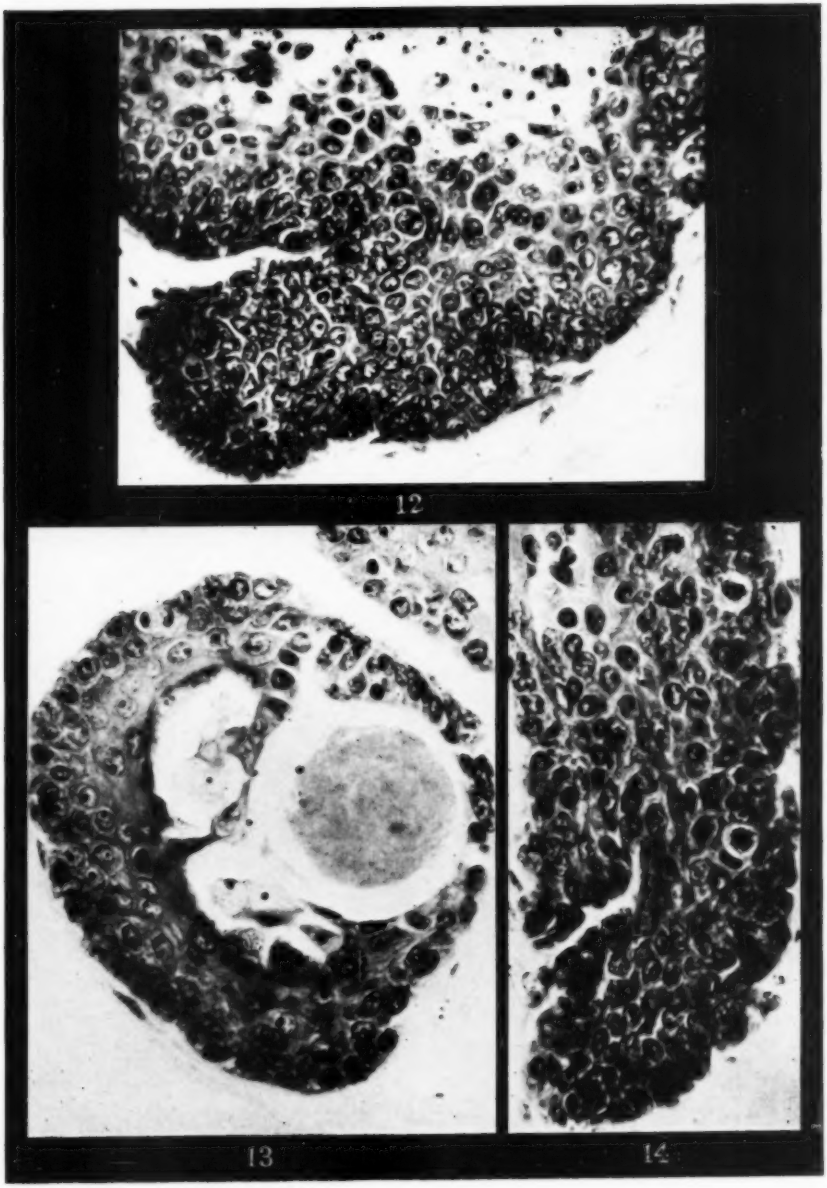

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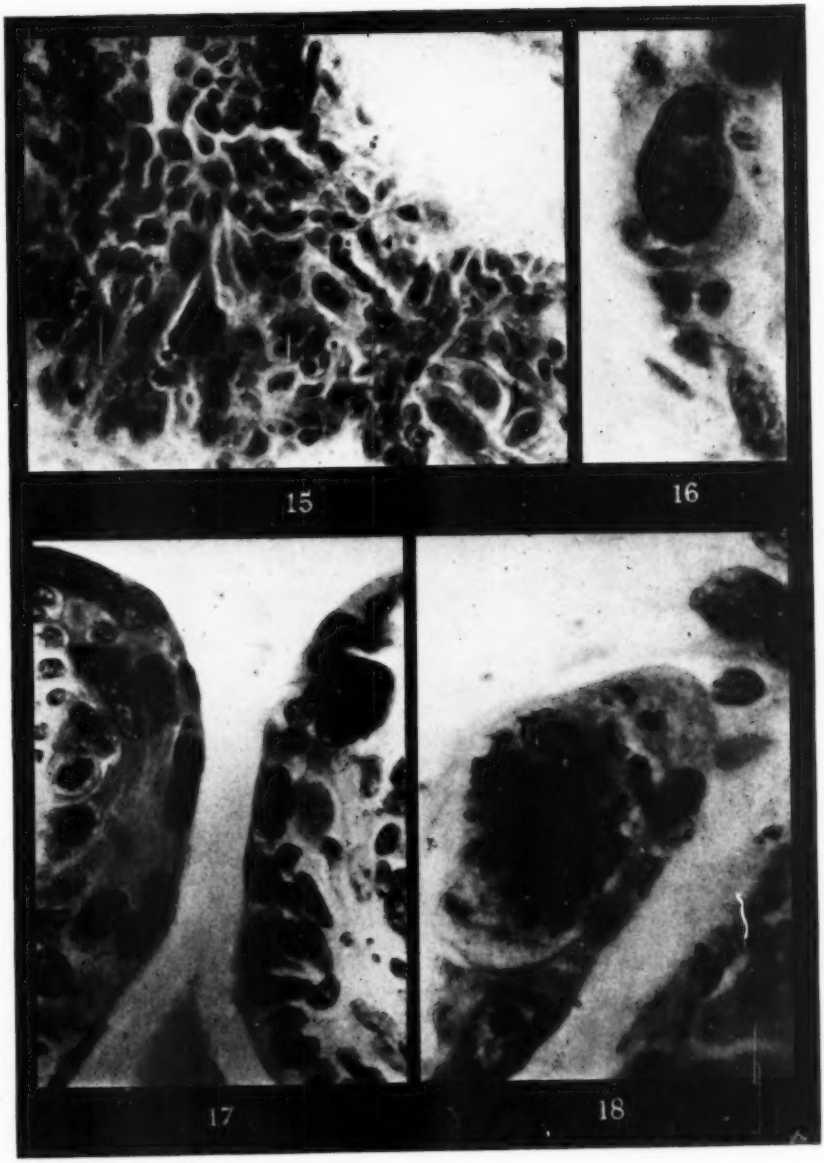

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The occurrence of abnormal mitotic figures, single very large cells and foci of anaplastic cells is in contrast to the more regular sequence : rise in cell division, hyperplasia, squamous metaplasia. The presence of abnormal mitosis and cell degeneration in this experiment suggests a damaging effect of the carcinogen, and a special sensitivity of the treated cells as compared with the stimulation seen in most cultures. It is reasonable to assume that the large mononucleate or multinucleate cells are derived from the polyploid mitotic cells, which thus become the ancestors of the anaplastic cells. The question arises as to which factor—direct stimulation by the carcinogen, or damage and somatic mutation of a few survivors (Haddow, 1947)—is responsible for an ultimate malignant transformation.

It is planned to investigate in future experiments whether the changes produced *in vitro* will be reversed on inoculation of the treated cultures *in vivo*, or whether they will persist and lead to malignancy.

The maintenance of glandular differentiation in treated cultures is interesting and can only be explained by the assumption that methylcholanthrene exerts on androgenic action, and thus replaces the gonadotropic hormones lacking in the culture medium.

SUMMARY.

Prostates of young mature mice were grown in watch-glasses in a medium of chick plasma, chick embryo extract and human serum.

In the experimental series 20-methylcholanthrene was added to the medium in a concentration of 2 gamma per c.c. and 4 gamma per c.c. The cultures were grown in the presence of the agent for 9 days, then transferred to a normal medium and fixed at intervals after removal of the carcinogen.

In both control and experimental cultures new alveoli were usually formed at the periphery of the explanted glands, while in the centre some of the alveoli underwent degeneration.

In the control cultures the epithelium quickly lost its glandular character, and the alveoli developed wide lumina lined by one layer of low epithelium in which mitosis was present though infrequent. The stroma, on the other hand, was dense.

In cultures treated with 2γ and 4γ/c.c. of methylcholanthrene, the alveolar epithelium retained its glandular character for a longer time and showed a marked increase in mitosis, leading to hyperplasia and squamous metaplasia, while the stroma was considerably reduced in contrast to that of the controls.

The first changes were recognizable 5 days after the beginning of treatment. They became more pronounced as the treatment continued and persisted after removal of the carcinogen. In the later stages partial or complete occlusion of the alveolar lumen with squamous metaplasia were observed.

The rise in mitosis showed two distinct peaks, the downward bend of the first wave coinciding with the removal of the carcinogen. The effect of the two concentrations was found to be qualitatively similar. After the higher dose, however, the increase in mitosis was delayed and slightly less than after the smaller concentration, while the appearance of squamous metaplasia was speeded up.

In some cultures abnormal mitotic figures, large mononucleate and multinucleate cells and foci consisting of anaplastic cells were observed.

It is concluded that methylcholanthrene in the doses used *directly* promotes the rate of cell division, causes epithelial hyperplasia and interferes with the normal process of differentiation, but that in some cases the same dose may exert a damaging effect according to the sensitivity of the treated cells.

I am indebted to Dr. Honor B. Fell for suggesting this problem and for her criticism in the preparation of the manuscript. I also wish to thank Dr. A. Howard, Radiotherapeutic Research Unit, Hammersmith Hospital, London, for the generous supply of C_3H males, Dr. C. B. V. Walker, Director of the Regional Blood Transfusion Centre, Cambridge, for providing human serum, and Mr. G. Lenney, who made the graph and microphotographs.

REFERENCES.

Cooper, Z. K., and Reller, H. C.—(1942) *J. nat. Cancer Inst.*, **2**, 335.
Creech, E. M. H.—(1940) *Amer. J. Cancer*, **39**, 149.
Dunning, W. F., Curtis, M. R., and Segaloff, A.—(1946) *Cancer Res.*, **5**, 256.
Earle, W. R.—(1943) *J. nat. Cancer Inst.*, **4**, 165.
Idem and Voegtlin, C.—(1938) *Amer. J. Cancer*, **34**, 373.
Fell, H. B., and Robison, R.—(1930) *Biochem. J.*, **24**, 1905.
Glucksmann, A.—(1945) *Cancer Res.*, **5**, 385.
Haddow, A.—(1947) *Brit. med. Bull.*, **4**, 331.
Horning, E. S., and Dmochowski, L.—(1947) *Brit. J. Cancer*, **2**, 59.
Moore, R. A., and Melchionna, R. H.—(1937*a*) *Amer. J. Cancer*, **30**, 731.—(1937*b*) *Amer. J. Path.*, **13**, 659.
Price, D.—(1949) *Ann. Rep. Strangeways Res. Lab., Cambridge*, p. 13.

THE NATURALLY OCCURRING EFFECT OF ZONES OF HAIR GROWTH ON EXPERIMENTAL CARCINOGENESIS IN THE RABBIT.

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HAVING observed that regeneration of hair in a clipped area on the back of a rabbit did not occur uniformly over the entire area, and occurred after varying intervals of time in different animals, it was decided to investigate the mode of regeneration of hair on the rabbit trunk. Hence, a band of hair, approximately 4 inches wide, completely encircling the trunk, was removed from a few rabbits, and it was observed that the depilated skin was not uniformly pink, but that there were roughly linear bluish-black bilaterally symmetrical zones. Besides these major dark zones, there were, in some animals, a few small irregular patches of a similar colour. The skin in these dark zones felt thicker than the rest of the skin. After two days, when the animals were inspected, it was found that there was prolific hair growth in the dark zones, which continued until the hair reached its normal length, but during all this time no hair growth was observed in the pink zones. We are at present studying this phenomenon in greater detail, as this method of hair growth is somewhat different from that in rats (Haddow, Elson, Roe, Rudall and Timmis, 1945), in which waves of hair growth spread around the trunk. Because of the rapid hair growth it would seem reasonable to suppose that there would be increased mitotic activity in the dark zones as compared with the pink ones. Consequently the application of a carcinogen to an area including both active and quiescent zones might result in a higher yield of tumours in the active zone, as it is well known that hyperplasia plays an important part in the genesis of neoplasms.

METHODS.

A band of hair approximately 4 inches wide, completely encircling the trunk, was removed from 10 agouti rabbits weighing between 1·2 and 1·8 kilos by initial clipping, followed by depilation with barium sulphide. Six of these were painted, on the left side from the mid-dorsal to the mid-ventral region, twice a week for the first 2 weeks, and then once a week for a further period of 12 weeks with 2 per cent W/W, 9 : 10-dimethyl-1 : 2-benzanthracene in lanoline, which was warmed to melting-point to facilitate application. The remaining 4 rabbits were treated in a similar manner with lanoline. Before each painting the hair which had grown was removed with electric clippers. The animals were photographed at the beginning of the experiment and on numerous subsequent occasions. Skin biopsies were taken at intervals from 3 experimental and 2 control animals. These were fixed in alcoholic Bouin and sections were stained with haematoxylin

and eosin. Each animal was kept in a separate metal cage without straw to prevent spread of the carcinogen to the non-painted side, and were fed *ad libitum* on the usual mixed diet with daily supplements of greens and turnips.

RESULTS.

(a) *Macroscopic appearance.*

Of the 6 experimental animals after the initial depilation, one showed a mid-dorsal active zone, two, bilaterally symmetrical lateral active zones (Fig. 1), two, mid-ventral active zones, and the remaining animal a lateral and a ventral zone. Of the 4 control animals, one showed a mid-dorsal active zone, two, a bilaterally symmetrical lateral active zone, and the remaining one showed no active zones at the beginning of the experiment but developed a mid-dorsal active zone within a fortnight.

As the experiment progressed the skin on the painted side of the experimental animals became darkened, thickened, and showed prolific hair growth until at the end of 30 days when the animals were photographed it was seen (Fig. 2) that the original zones were completely masked by vigorous hair growth over the entire painted side. During this time there was suppression of activity in the non-painted sides with disappearance of active zones in 5 out of the 6 experimental animals and the entire side became completely bald, activity ceasing in the sixth animal about a week later.

The first papilloma appeared on the 53rd day (Fig. 3) in an initial growth zone (Fig. 1) and all the animals showed one or more papillomata by the 71st day, and in each case the first papilloma appeared in an area corresponding to an initial growth zone (Fig. 1 and 3), but subsequent papillomata appeared in a non-selective fashion over the entire painted area except that at any given time in every animal there were larger numbers of tumours in the originally active zone than in the quiescent ones (Fig. 4). With the appearance of the papillomata changes began to occur in the surrounding skin ; the hair on the painted side now failed to show the vigorous growth it had shown so far. The skin felt thinner, but the whole zone still showed a dark, bluish-black pigmentation. On the un-painted side, which had been quiescent previously, bluish-black zones of activity appeared, followed by hair growth. On the 82nd day it was observed that one of the papillomata in an originally active zone seemed to have undergone a malignant change. A biopsy was taken on the 89th day, and this showed it to be a squamous cell carcinoma (Fig. 12). In the control series no difference in the rate of hair growth or distribution of actual zones was seen between the painted and non-painted sides.

(b) *Microscopical appearance.*

To determine the normal histology of the skin, biopsies were taken from several areas of the active and quiescent zones in two of the control animals before painting with lanoline. Considerable difference was observed between these two zones. In the pink quiescent zone (Fig. 5) the epidermis was only one or two cells thick covered with a fine layer of keratin. The hair follicles were scanty, and did not extend deeply into the dermis. There was a very small amount of melanin pigment in the basal layer. In the active zone, on the other hand, the epidermis was thicker and the layer of keratin more prominent. The hair follicles were extremely prominent and extended deeply into the dermis.

The follicles showed some arrangement into groups, and there was a greater amount of melanin pigmentation in the basal layer of the epidermis and in the hair follicles (Fig. 6).

The skin, after 6 applications of carcinogen, showed considerable change (Fig. 7). As already stated, by this time the whole of the skin on the painted side had attained a uniform appearance, and no distinction between active and quiescent zones was seen. The epidermis was much thicker than either the active or quiescent skin before painting. This change appeared to extend down into the follicles, which were more prominent and deeply seated and showed the usual arrangement in groups. The epidermis was hyperkeratotic, and showed increased pigmentation in the basal layers and the hair follicles. Phagocytes laden with the same pigment were present in the dermis (Fig. 8). The skin on the non-painted side of the experimental animal, which by now showed no active zones, presented a similar picture to the inactive zones before painting (Fig. 9).

After the papillomata appeared, sections of the surrounding skin (Fig. 10) on the painted side still showed hyperkeratosis and considerable thickening and pigmentation of the epidermis. However, there was a reduction in the number of hair follicles, which extended only a short way into the dermis. These were considerably distorted, and there were also small blunt down-growths of epidermis into the dermis.

DISCUSSION.

The close association between hyperplastic states and the development of tumours has been stressed by various authors. Willis (1948) states: " In the skin epidermal hyperplasias evoked by various irritative and inflammatory lesions sometimes become cancerous . . . carcinoma of the prostate frequently arises in an organ already the seat of benign enlargement."

It is well known that carcinomata are of more frequent occurrence than sarcomata. It is possible that one of the factors responsible for this may be the normally occurring higher mitotic rate of epithelia as compared with connective tissue. Conversely, cells such as skeletal muscle and nerve cells which show little or no division during adult life infrequently undergo neoplastic transformation. Similarly, experimental evidence tends to show that agents which stimulate mitotic activity promote tumour production. Mottram (1944*a*, 1944*b*) showed that the skin of mice can be sensitized by painting with croton oil, so that a subsequent single application of 3 : 4-benzpyrene leads to an abundant production of tumours and a single application of carcinogen followed by repeated applications of croton oil also produces tumours. Hence Mottram (1944*b*) concluded that carcinogenesis was composed of three phases: (1) a " sensitizing factor " which could be brought about by preliminary treatment with croton oil, acting presumably in a non-specific manner by causing hyperplasia, on the supposition that proliferating cells were more responsive than resting cells to the specific carcinogenic action which followed. (2) A " specific cellular reaction " induced by the specific action (even of a single application) of a carcinogen, this representing the essential neoplastic change. (3) A " developing factor " responsible for the actual appearance of a visible wart, and produced by croton oil of by a carcinogen. Berenblum and Shubik (1947*a*, 1947*b*) confirmed the latter part of his observations, but found no evidence to suggest that a preliminary croton oil hyperplasia had an augmenting influence on tumour production, which would

seem to eliminate Mottram's (1944*b*) sensitizing factor. They suggested that the initial action in carcinogenesis constitutes a sudden and irreversible process whereby a few normal cells are changed into permanently altered latent tumour cells, which lie dormant till stimulated into activity by a promoter such as croton oil. Croton oil has been shown by Bullough (1950) to be a mitotic stimulant in the mouse epidermis: conversely Tannenbaum (1947), using mice in experiments involving restriction of diet, found that if animals were maintained on a 66 per cent diet they developed fewer tumours than the fully-fed controls. Bullough and Eisa (1950) have shown that a reduction of the diet to 70–80 per cent of normal is followed by an optimum depression in the epidermal mitotic rate in mice.

All this evidence tends to show that the genesis of tumours is closely related to the mitotic rate in the parent tissue.

In the rabbit we have an animal in which there are adjacent areas of the skin of markedly different activity, dark zones showing intense hair growth and therefore presumably a heightened mitotic activity and pale pink zones of low activity. As the tumours appeared earlier and in larger numbers in the former areas than in the latter, our experimental results tend to support the hypothesis that genesis of tumour production is related to the mitotic activity in the parent tissue. It is obvious that as the increased mitotic rate must have continued for a considerable time after the first painting, the naturally occurring increased mitotic activity had acted as a promoting agent. Further, if the carcinogen produces the latent neoplastic change in the follicles as well as in the epidermis, and as the increased mitotic rate during hair growth is mainly confined to the follicles, it would seem feasible that the tumours originated in the follicles.

As many common solvents, such as benzene, cause an inflammatory reaction and an increase in mitotic activity, and thus possibly act as promoting agents

EXPLANATION OF PLATES.

FIG. 1.—Appearance of experimental animal after depilation and before painting, showing one of the bilaterally symmetrical major zones (A) of active hair growth, and two small minor zones of activity (B and C).

FIG. 2.—Dorsal view of same animal as Fig. 1 after 6 paintings with 9 : 10-dimethyl-1 : 2-benzanthracene, on the left side showing masking of previous zones, prolific hair growth on the painted side and suppression of hair growth on the contralateral side.

FIG. 3.—Photograph of same rabbit as in Fig. 1 after 9 paintings with 9 : 10-dimethyl-1 : 2-benzanthracene showing initial tumour (X) which has occurred in the originally active zone (Fig. 1, A) and a second tumour (Y) which seems to have occurred in an originally small active minor zone (Fig. 1, B).

FIG. 4.—The same animal as in previous photographs, showing preponderance of papillomata in the originally active as compared with quiescent zones (Fig. 1).

FIG. 5.—Section of skin from quiescent zone before painting. Note the scanty hair follicles and thin epidermis. × 45.

FIG. 6.—Section of skin from active zone before painting showing thicker epidermis and groups of hair follicles extending deeply into the dermis. × 45.

FIG. 7.—Section of skin from the painted side after 6 applications of 9 : 10-dimethyl-1 : 2-benzanthracene. Note hyperplastic changes in the epidermis extending into the hair follicles. × 45.

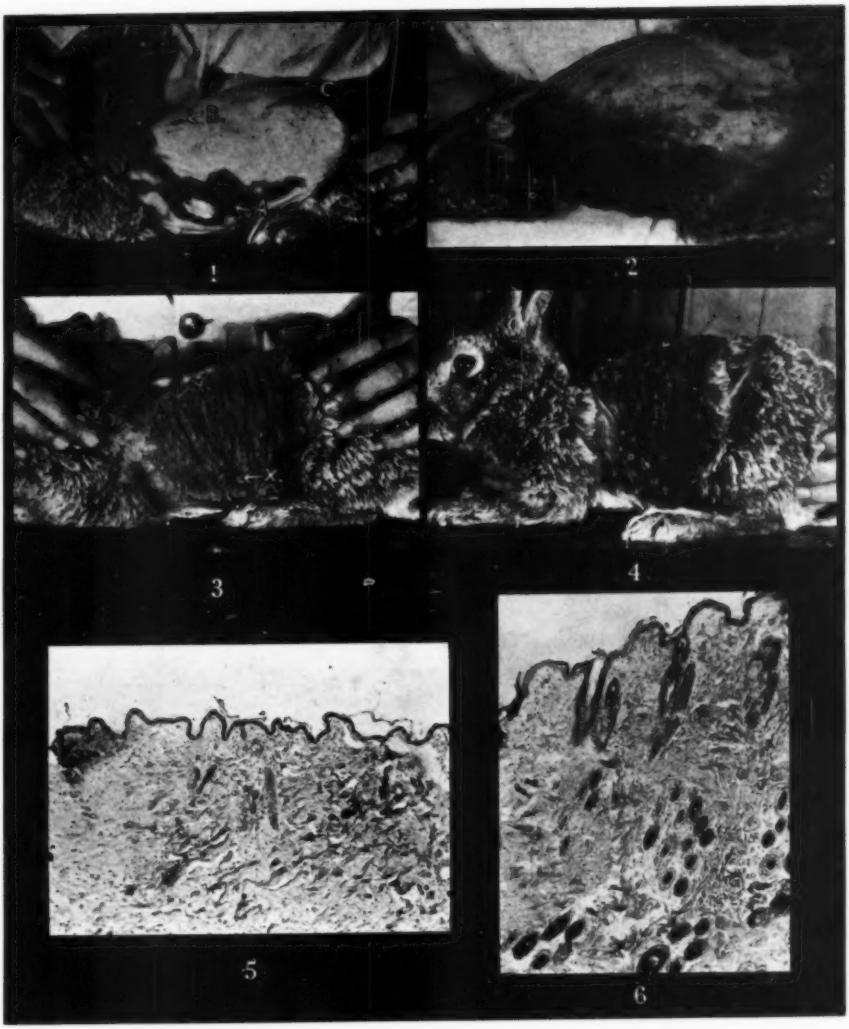
FIG. 8.—High-power view from Fig. 7 showing increased pigmentation in epidermal cells and melanophores in the dermis. × 180.

FIG. 9.—Skin from the non-painted side of an experimental animal (Fig. 7). × 45.

FIG. 10.—Skin from painted side after twelve paintings with 9 : 10-dimethyl-1 : 2-benzanthracene 18 days after appearance of the first papilloma. × 65.

FIG. 11.—Papilloma. × 6.

FIG. 12.—Biopsy from edge of malignant ulcer. × 15.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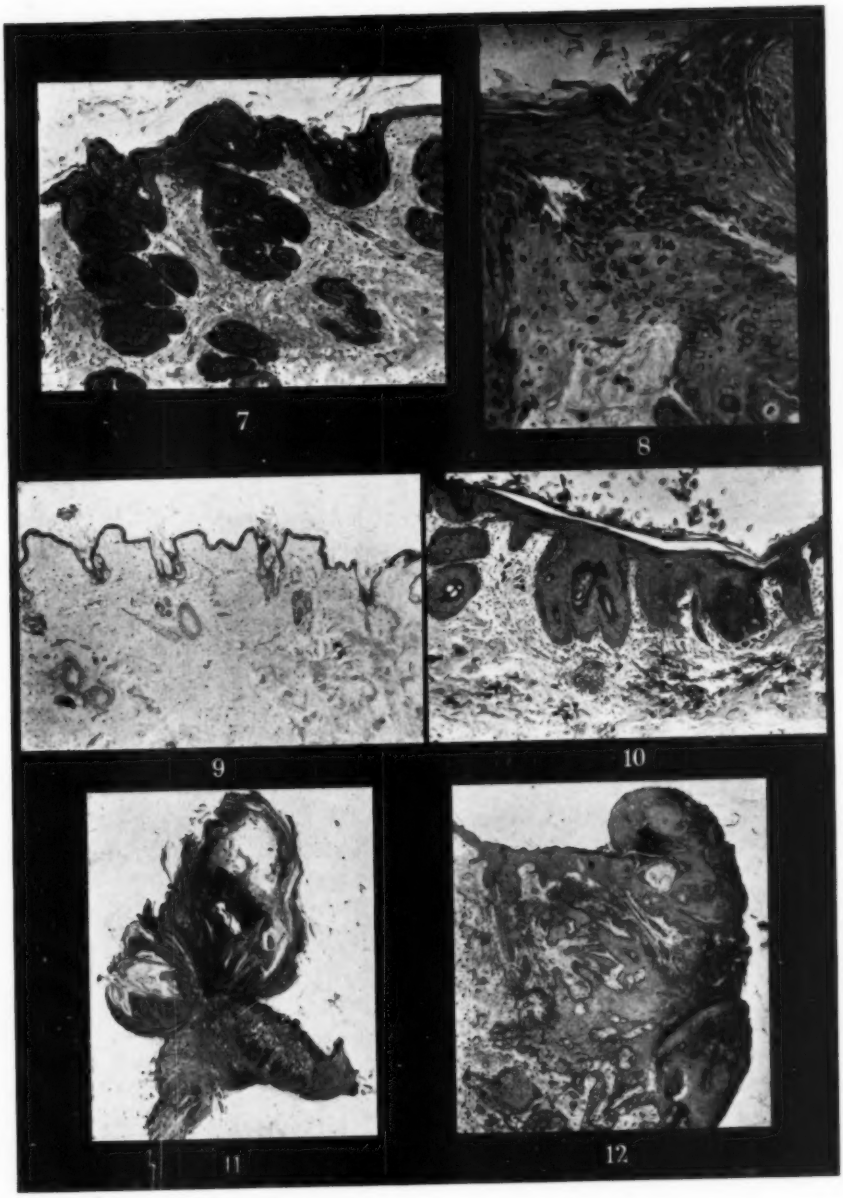

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lanoline was selected as a solvent, as it is a relatively inert substance in the sense that it produces no inflammatory reaction, and would not cause masking of the naturally occurring zones of activity.

SUMMARY.

A band of hair 4 inches wide completely encircling the trunk was removed from 10 rabbits. The depilated skin showed bilaterally symmetrical dark zones which showed prolific hair growth within a few days, the rest of the skin remaining bald. 9 : 10-Dimethyl-1 : 2-benzanthracene in lanoline was painted on the left half of 6 animals; the remaining 4 were painted with lanoline. The following changes were observed:

(1) Within 30 days the painted area in the experimental animals became dark and showed prolific hair growth, while on the opposite side there was suppression of hair growth, the skin becoming bald.

(2) In all animals initial papillomata appeared at the site of an originally active zone. The first papilloma appeared after 53 days.

(3) After the appearance of papillomata hair growth ceased and zones of activity appeared on the opposite side.

(4) The first carcinoma appeared after 81 days at the site of an originally active zone.

It was concluded that (*a*) the naturally occurring zones of increased mitotic activity which continued for some time after the first painting must have acted as a promoting agent; (*b*) it seems more probable that the tumours arose from hair follicles, and not the epidermis, as the former are the site of increased mitotic activity during hair growth.

It gives us great pleasure to express our thanks to Professor H. N. Green for his active interest and constructive criticism during the course of these experiments.

REFERENCES.

Berenblum, I., and Shubik, P.—(1947*a*) *Brit. J. Cancer*, **1**, 379.—(1947*b*) *Ibid.*, **1**, 383.

Bullough, W. S.—(1950) *Ibid.*, **4**, 329.

Idem and Eisa, E. A.—(1950) *Ibid.*, **4**, 321.

Haddow, A., Elson, L. A., Roe, E. M. F., Rudall, K. M., and Timmis, G. M.—(1945) *Nature*, **155**, 379.

Mottram, J. C.—(1944*a*) *J. Path. Bact.*, **56**, 181.—(1944*b*) *Ibid.*, **56**, 391.

Tannenbaum, A.—(1947) 'Approaches to Tumour Chemotherapy.' Washington (Amer. Ass. Adv. Sci.).

Willis, R. A.—(1948) 'Pathology of Tumours,' p. 124. London (Butterworth).

A BASIS FOR THE SELECTION OF CARCINOGENS LIKELY TO PRODUCE EXPERIMENTAL GASTRIC CANCER.

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THE experimental investigation of gastric cancer is seriously impeded by our present inability readily to produce this condition in animals. Some gastric cancer has been initiated by the implantation of carcinogenic hydrocarbons directly in the stomach wall. The difficulty and uncertainty of this method have precluded its general use. Progress in this field would be greatly facilitated if a chemical compound capable of producing gastric cancer were discovered. That a carcinogen with such specificity is possible may be implied from the frequency with which Butter Yellow, for example, produces hepatomas (Kinosita, 1940) and beta-napthylamine, bladder tumors (Wiley, 1938). The obvious approach would be to incorporate a carcinogen in the diet. When this is done it very seldom results in malignant growth in the stomach because of the protective action of the mucus. If the carcinogen could be *secreted* by the stomach it would be in a much more favourable position to initiate cancer.

The first step towards a solution of the problem is to ascertain just what properties of a substance influence its secretion by the glands of the stomach. The optimum properties might then be conferred on a carcinogen by judicious substitution in the molecule. The resulting compound could be expected to initiate neoplastic growth in the stomach.

Several groups of workers during the past twenty-five years have studied, by means of gastric pouches in dogs, the secretion of intravenously injected dyes. Dawson and Ivy (1925), Kobayashi (1926), Ingraham and Visscher (1935), and Varco and Visscher (1941), although working under varied conditions, are generally in agreement as to the dyes secreted by the stomach. Of some sixty dyes studied (Ingraham and Visscher, 1935), all those secreted are electropositive (basic) under some conditions. On the other hand, dyes not secreted by the gastric glands are acid or amphoteric with the possible exception of four basic triphenylmethane dyes.

The basicity of these dyes seems to be the property most likely to determine their *degree* of gastric secretion. We have, therefore, determined the pK_B of those dyestuffs whose concentration ratios (concentration of dye in gastric juice : concentration in blood) are known.

EXPERIMENTAL METHODS.

Basic ionization constants were determined by potentiometric titration. A Beckman, Model G, pH meter was employed with Glass Electrode 1190T and Calomel Electrode 1170. The Beckman 1190E blue glass electrode was used when the pH rose above 10. The electrodes were standardized before each titration against buffer solutions of pH 7 or pH 10. All titrations were carried out in a room maintained at a temperature of $25° \pm 1°$.

In general, the dyes studied were not strong bases. In most cases the hydrochloride (sulfate in the case of Nile Blue) salt was titrated with 0·048 N sodium hydroxide. Samples of 0·1200 to 0·2500 g. of the salt were dissolved in 300 ml. of water to correspond to a molarity of 0·0013 to 0·0027. Eastman (1925) has calculated that for a 0·001 molar acid titration with a strong base the titration curve will not yield an inflection at the end-point if K_A is less than 10^{-8}. This we found to be true. Such compounds were titrated as the free base with 0·047 N hydrochloric acid.

The values for pK_B were calculated from at least five points between 25 and 75 per cent neutralization. As an example, the calculation of K_B for the dye Rhodamine B is given. The titration curve (Fig. 1) indicates an end-point at

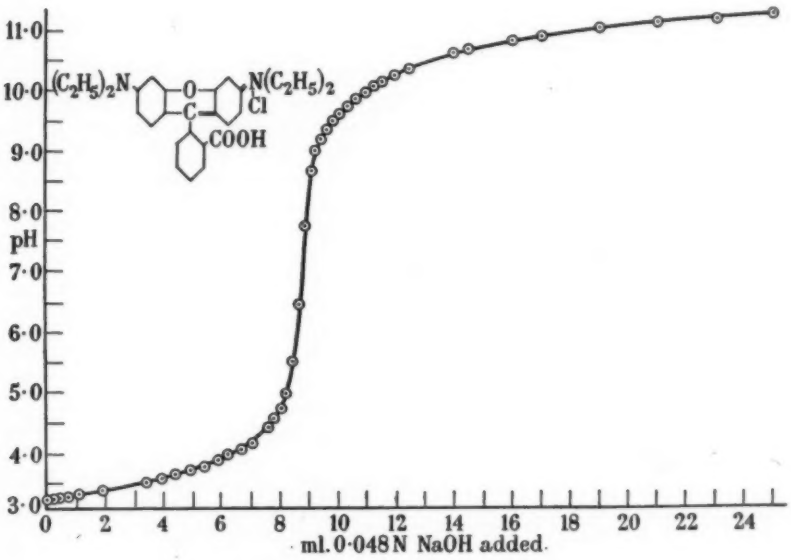

FIG. 1.—Potentiometric titration curve of Rhodamine B.

8·8 ml. of 0·048 N sodium hydroxide using 300 ml. of solution. After the addition of 4·5 ml. of sodium hydroxide a pH reading of 3·60 was obtained. This corresponds to a hydrogen ion concentration of $2{\cdot}51 \times 10^{-4}$ millimoles per ml.

$BH^+_{(original)} = 8{\cdot}8\ (0{\cdot}048) = 0{\cdot}422$ millimoles.
$B.HCl + HOH \rightleftarrows B.HOH_{(hydrolysis)} + H^+ + Cl^-$, therefore,
$B.HOH_{(hydrolysis)} = 300$ ml. $(2{\cdot}51 \times 10^{-4}$ millimoles/ml.$) = 0{\cdot}075$ millimoles.
$B.HOH_{(neutralization)} = 4{\cdot}5\ (0{\cdot}048) = 0{\cdot}216$ millimoles.
$B.HOH_{(total)} = 0{\cdot}075 + 0{\cdot}216 = 0{\cdot}291$ millimoles.
$BH^+ = 0{\cdot}422 - 0{\cdot}291 = 0{\cdot}131$ millimoles.

$$K_B = \frac{(BH^+ \mid 300)K_W}{(B.HOH \mid 300)(C_{H^+})} = \frac{0{\cdot}131 \times 10^{-14}}{0{\cdot}291\ (2{\cdot}51 \times 10^{-4})} = 1{\cdot}79 \times 10^{-11}.$$

These calculations were repeated at various points throughout the titration. Results are tabulated in Table I.

TABLE I.—*Ionization Constants Calculated for Rhodamine B.*

	Per cent neutralization					
	23.	40.	46.	51.	63.	74.
$K_B \times 10^{11}$	1·68	1·75	1·70	1·79	1·71	1·88
pK_B	10·77	10·76	10·77	10·75	10·77	10·73

The average value for K_B of each of the dyes studied is given in Table II, column 2.

DISCUSSION.

Derivation of a formula expressing the concentration ratio of compounds in stomach and blood as a function of their pK_B.

If we assume a simple distribution mechanism to explain the concentration of basic dyes in the stomach, the concentration ratio may be calculated from the ionization constant of the dye and the hydrogen ion concentrations of gastric juice and blood. Assume these two solutions to be well buffered at a pH of 1 and 7·4 respectively and separated by a membrane freely permeable to the undissociated dye. In both solutions the total concentration of dye will be equal to that of the dissociated and undissociated forms.

TABLE II.—*Calculated and Experimental Concentration Ratios.*

Dye.	pK_B.	$\frac{C_S}{C_B}$ (Kobayashi, 1926).	$\frac{C_S}{C_B}$ (calculated).
Nile blue	11·6	0·8	1·5
Thioflavine T	11·3	1·3	2·8
Acridine red	10·9	8·4	6·1
Rhodamine B	10·8	6·1	7·3
Brilliant cresyl blue	10·8	11·3	7·3
Methylene green	10·8	13·6	7·3
Methylene blue	10·2	15·6	16·8
Bismark brown	9·0	26·5	28·6
Chrysoidine Y	8·7	75·0	29·0
Safranin O	7·6	27·3	27·3
Neutral red	7·5	26·4	26·5
Thionin	7·1	36·8	22·7
Toluidine blue	6·5	36·4	13·3
Pyronine B	6·3	5·7	10·0

$$\text{Total dye} = [B.HOH] + [BH^+].$$

The quantity $[BH^+]$ may be expressed by

$$[BH^+] = \frac{K_B}{10^{-14}} [H^+] [B.HOH].$$

The total dye in either solution is then given by

$$\text{Total dye} = [\text{B.HOH}]\left\{1 + \frac{K_B}{10^{-14}}[H^+]\right\}.$$

The concentration ratio then between gastric juice, with a pH of 1, and blood, with a pH of 7·4 may be expressed by

$$\frac{C_S}{C_B} = \frac{[\text{B.HOH}]_S\left\{1 + \frac{K_B}{10^{-14}}(10^{-1})\right\}}{[\text{B.HOH}]_B\left\{1 + \frac{K_B}{10^{-14}}(10^{-7\cdot4})\right\}}.$$

Assume $[\text{B.HOH}]$ in stomach $= [\text{B.HOH}]$ in blood.

Then

$$\frac{C_S}{C_B} = \frac{1 + 10^{13}\,K_B}{1 + 10^{6\cdot6}\,K_B}.$$

Substituting the experimentally determined ionization constants in this equation, concentration ratios are obtained which are far higher than those realized by Ingraham and Visscher (1935). At a pK_B of 11·3, for instance, this equation yields a concentration ratio of 51, at a pK_B of 7·5 a ratio of $2{\cdot}8 \times 10^5$. Ratios actually obtained were 1·3 and 26·4. Although experimental results indicate a definite tendency for secretion to increase with increasing basicity it is obvious that there is some opposing factor. This opposing factor increases with increasing basicity, so that at pK_B of about 6·0 the tendency for the basic dye to concentrate in the stomach, due to distribution differences, might be completely overcome.

This opposition to gastric secretion of dyes of increasing basicity is nicely explained in terms of the pore theory, or the concept of the positively charged membrane (Ingraham and Visscher, 1935). Acid dyes would be retained by chemical combination. Basic dyes are assumed to pass through this membrane in the undissociated form. As the ionization constants of these dyes increase it would become increasingly more difficult for them to pass through a positively charged membrane.

It appears, therefore, that we are dealing with two main effects in the gastric secretion of dyes. One arises from the tendency of basic dyes to accumulate in the stomach because of the pH difference between blood and gastric juice. The other is possibly due to the existence of a positively charged membrane, impermeable to acid dyes but permeable to the undissociated basic dye. As the basic dyes become stronger the charged membrane tends to restrain them, and their secretion in the stomach is thereby inhibited.

Such restraint of basic dyes and consequent inhibition of their secretion should be directly related to the ionization constants of the dyes. It should be possible then to obtain a factor, involving the ionization constant of the dye, which would account for this effect opposing secretion. Such an empirical factor was devised experimentally.

$$\frac{3 \times 10^{-12}}{K_B + 5 \times 10^{-11}}.$$

This factor, when multiplied by the concentration ratios calculated through assumption of a simple distribution mechanism,

$$\frac{C_S}{C_B} = \frac{1 + 10^{13}\ K_B}{1 + 10^{6.6}\ K_B} \cdot \frac{3 \times 10^{-12}}{K_B + 5 \times 10^{-11}},$$

gave concentration ratios closely following those reported by Ingraham and Visscher (1935) (Table II, Column 4). In view of the possibilities for error in determining the experimental concentration ratios (Kohn, Komarov and Shay, 1949), the correlation (Fig. 2) between experimental and calculated values appears sufficient to justify, tentatively, the use of the formula.

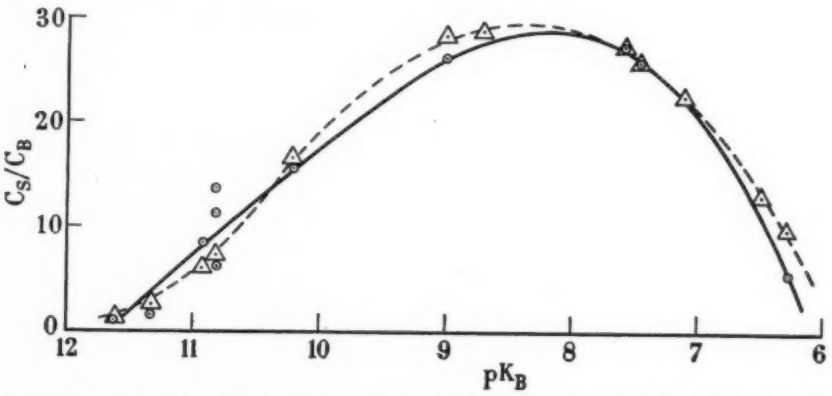

FIG. 2.—Curves showing both the experimental and calculated values of pK_B plotted against the concentration ratios.

Chrysoidine Y $C_S/C_B = 75$ (Kobayashi, 1926).
△ - - - △ = calculated.
⊙ —— ⊙ = experimental.

By means of this formula it should be possible to predict which compounds will be secreted in the stomach and to what extent. A pK_B within the range 7·5 to 9·0 is indicated for maximum secretion. A carcinogen may now, by a variation of substituents, be adjusted to a pK_B within this range. It is not unreasonable to expect such compounds, through localization in the stomach over a considerable period of time, to initiate neoplastic growth in this organ. Soluble derivatives of the carcinogen, 2-aminofluorene with pK_B falling within the range 7·5 to 9·0 have been prepared in this laboratory and are now under test.

SUMMARY.

Two concepts of gastric secretion of dyes, the positively charged membrane, and the distribution difference between blood and gastric juice, have been integrated and a mathematical formula developed. It is suggested that this will serve, at least tentatively, as a basis for the selection of carcinogenic compounds that will be secreted by the stomach and initiate neoplastic growth in that organ.

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REFERENCES.

DAWSON, A. B., AND IVY, A. C.—(1925) *Amer. J. Physiol.*, **73**, 304.
EASTMAN, E. D.—(1925) *J. Amer. chem. Soc.*, **47**, 332.
INGRAHAM, R. C., AND VISSCHER, M. B.—(1935) *J. gen. Physiol.*, **18**, 695.
KINOSITA, R.—(1940) *Yale J. Biol. Med.*, **12**, 287.
KOBAYASHI, K.—(1926) *Acta Sch. med. Univ. Kioto*, **8**, 465.
KOHN, R., KOMAROV, S. A., AND SHAY, H.—(1949) *Rev. Canad. Biol.*, **8**, 262.
VARCO, R. L., AND VISSCHER, M. B.—(1941) *Proc. Soc. exp. Biol. N.Y.*, **46**, 295.
WILEY, F. H.—(1938) *J. biol. Chem.*, **124**, 627.

SECRETION BY THE STOMACH OF POTENTIAL CARCINOGENS DERIVED FROM 2-AMINOFLUORENE

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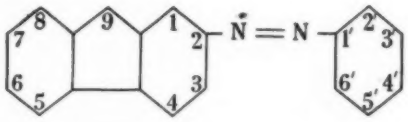
IN the preceding paper Ray and Jung (1951) developed certain criteria for the selection of compounds likely to be secreted by the stomach. In general, compounds with a pK_B value between 7 and 10 seemed to be secreted by the stomach in very appreciable amounts. The ratio of the concentration of the compounds in the stomach to that in the blood in this range was about 20 : 1 or greater.

By selecting carcinogens that have a pK_B falling in this range it should be possible to circumvent the mucus barrier and expose the glandular stomach to attack. With this in mind a series of derivatives of 2-aminofluorene were prepared. The carcinogenicity of 2-aminofluorene and several of its derivatives has been abundantly established by many workers, and the literature has been recently reviewed by Morris, Dubnik and Johnson (1950).

In the present work 2-aminofluorene was diazotized and coupled with a number of phenols and amines to form azo compounds (Table I). The azo linkage was selected for several reasons. Firstly, Dawson and Ivy (1925)



reported that Chrysoidine Y (2,4-diaminoazobenzene) appears in the gastric juice in dogs 5 minutes after intravenous injection. Secondly, many azo dyes have been shown to be carcinogenic or cocarcinogenic; Butter Yellow and its derivatives causing hepatomas; 4-hydroxy azobenzene causing a high incidence of papillomas of the stomach of rats (Kinosita and Harada, 1938); and Chrysoidine R (5-methyl-2,4-diaminoazobenzene) promoting tar-induced cancers (Forssman, 1931). Thirdly, *in vivo* metabolism studies of Butter Yellow have revealed the splitting of the azo link, each nitrogen atom being reduced to an amino group (Williams, 1947). Thus, the splitting of the azo compounds of fluorene would be expected to produce an active carcinogen, 2-aminofluorene.

TABLE I.—*Compounds Derived from 2-Aminofluorene.*

Compound.	Formula.	Melting-point.† Obtained.	Melting-point.† Previous.	Analysis.* Carbon per cent. Calc.	Carbon per cent. Fd.	Hydrogen per cent. Calc.	Hydrogen per cent. Fd.	Nitrogen per cent. Calc.	Nitrogen per cent. Fd.
I	R-C_6H_4-4′-$N(CH_3)_2$	d. 227	d. 227‡	80·47	80·39	6·11	6·25	13·41	13·41
II	R-C_6H_4-4′-OH	d. 197–198	d. 187·5–191§	79·69	79·64	4·92	4·45	9·78	9·88
III	R-C_6H_3-2′,4′-$(OH)_2$	d. 216	d. 204–204·5§	75·47	75·64	4·66	4·88	9·26	9·16
IV	R-C_6H_3-2′-CH_3-4′-OH	d. 210	d. 200§	79·97	79·75	5·36	5·53	9·32	9·39
V	R-C_6H_3-2′,4′-$(NH_2)_2$	d. 215	—	76·00	76·14	5·37	5·61	18·66	18·63
VI	R-C_6H_3-5′-CH_3-2′,4′-$(NH_2)_2$	d. 236	—	76·40	76·24	5·77	5·54	17·82	17·99
VII	R-C_6H_3-2′-NH_2-4′-OH	d. 194–5	—	75·72	75·39	5·01	5·07	13·94	13·93

* Analyses performed by Oakwold laboratories, Alexandria, Virginia and Galbraith Laboratories, Knoxville, Tennessee.
† All m.p. uncorrected.
‡ Korczynski, Karlowski and Kierzik. (1927).
§ Bielenberg, Goldhahn, and Pluskal (1940).

R = —N = N—

TABLE II.—*Compounds Derived from 2-Acetylamino-7-aminofluorene.*

Compound.	Formula.	Melting-point.	Analysis.* Carbon per cent. Calc.	Carbon per cent. Fd.	Hydrogen per cent. Calc.	Hydrogen per cent. Fd.	Nitrogen per cent. Calc.	Nitrogen per cent. Fd.
IA	R″-C_6H_3-2′,4′-$(NH_2)_2$	d. 269	70·56	70·50	5·35	5·70	19·59	19·73
IIA	R″-C_6H_3-5′-CH_3-2′,4′-$(NH_2)_2$	d. 241–242	71·13	70·98	5·68	5·86	18·85	19·07
IIIA	R″-C_6H_4-4′-$N(CH_3)_2$	d. 260	74·56	74·28	5·98	5·60	15·12	15·28
IVA	R″-C_6H_3-2′,4′-$(OH)_2$	d. 288–9	70·17	69·65	4·76	4·83	11·69	11·22
VA	R″-C_6H_3-2′-NH_2-4′-OH	d. 253	70·37	70·48	5·06	5·10	15·63	15·38

* See note to Table I.

R″ = CH_2CONH— —N = N—

Because " more tumors have been induced by 2,7-diacetyldiaminofluorene than by 2-acetylaminofluorene" (Morris and Dubnik, 1950), a second series of fluorene azo compounds was prepared by diazotizing and coupling 2-acetylamino-7-aminofluorene with several phenols and amines (Table II).

EXPERIMENTAL TECHNIQUES.

Three compounds selected from the first series were tested on rats. They were fluorene-2-azo-2′,4′-dihydroxy benzene, Cpd. III; fluorene-2-azo-2′-methyl-4′-hydroxy benzene, Cpd. IV; and fluorene-2-azo-5′-methyl-2′,4′-diamino benzene, Cpd. VI. Rats of the Sprague-Dawley-Holtzman strain weighing 120 to 280 g. were injected intraperitoneally with 2·5 mg. of the compounds dissolved in propylene glycol (Table III). Three hours previously these animals had been operated

TABLE III.—*Conditions of Administration.*

Compound.	Color.	Absorption maximum in mμ	pK_B.	Dose.		Animal weights (g.).		
				Propylene glycol (ml.).	Compound (mg.).	1 hr.	0·5 hr.	0·25 hr.
III	Red crystals	400	10·4	2	2·5	280	120	165
IV	Brown yellow crystals	380	10·3	1·5	2·5	235	120	—
VI	Red crystals	430	8·5	1·5	2·5	210	133	187
Control	—	—	—	1·5	—	—	—	175

on and a ligature placed below the pylorus to prevent regurgitation from the intestine. The animals had been fasted for 48 hours preceding the operation to avoid contamination of the secretions with food. Simultaneously with the injection of the compounds to be tested 0·2 mg. per 100 g. body weight of histamine was injected subcutaneously to assure an adequate flow of gastric juice. The animals were killed at 1 hour, 0·5 hour and 0·25 hour following injections; the stomachs were removed and the contents collected.

The stomach contents were diluted with an equal volume of acetone, stirred thoroughly and centrifuged for 10 minutes at 2000 r.p.m. One ml. of supernatant was diluted to 10 ml. with acetone and this extract allowed to stand overnight at 5–10° C. It was then centrifuged for 20 minutes at 2000 r.p.m. and the supernatant used for spectrographic analysis. Measurements were made on a Beckman Model DU Spectrophotometer against acetone-HCl standard having the same pH as the gastric extracts. Standard curves were obtained which essentially followed Beer's Law. The control was ligated and injected with histamine and prophylene glycol exactly as described for the experimental animals.

Preparation of compounds.

The possibility of the formation of fluorene-azo dyes was postulated in 1901 by Diels. Since then several of these compounds have been reported (Korczynski, Karlowski and Kierzik, 1927; Bielenberg, Goldhahn and Pluskal, 1940).

The starting material, fluorene, m.p. 111–112° C., was nitrated and reduced according to procedures given by Blatt (1943), and the amine diazotized forming fluorene-2-diazonium chloride dihydrate (Adams, 1944; Diels, 1901).

The dyes of Table I were prepared by coupling the components in acid buffered or basic aqueous solution, neutralizing and filtering. All could be recrystallized from dilute acetone, but for highest purity the amino derivatives were recrystallized from aniline and the hydroxy derivatives from dilute acetic acid or benzyl alcohol. Yields ranged from 58 to 87 per cent. Constants and analyses are given in Table I. It will be noted that three are new compounds and that three of those previously prepared have higher decomposition points, indicating probable improvement in quality. Previously only nitrogen analyses were reported.

For the preparation of the " A " series of dyes (Table II) 2-nitrofluorene was further nitrated at room temperature with yellow fuming nitric acid in glacial acetic acid (Anastakrishnan and Hughes, 1935). The resulting 2,5- and 2,7-dinitrofluorenes were separated by refluxing for 1 hour in glacial acetic acid and filtering hot. The 2,5-isomer is soluble, whereas the 2,7-isomer is not (Morgan and Thomason, 1926). Yields of 55 to 60 per cent were obtained of 2,7-dinitrofluorene decomposing at 293–4°.

The 2,7-dinitrofluorene was reduced with tin and hydrochloric acid by the method of Schulman (1949). The pure compound melts at 165° after recrystallization from benzene. This was then converted to the dihydrochloride and mono-acetylated in aqueous solution (Schulman, 1949). 2-Acetylamino-7-aminofluorene hydrochloride was then slurried in 500 ml. of water containing 7·5 ml. of concentrated hydrochloric acid with the aid of a Waring blendor which materially aided the diazotization of this rather insoluble compound. A solution of sodium nitrite (2·4 g. in 30 ml. water) was added to the rapidly stirred suspension over the period of one hour. The orange red suspension was heated to 50–60°, filtered, and the cooled filtrate treated with 150 g. of sodium chloride to precipitate the diazonium salt. (In coupling procedures it was not necessary to isolate the diazonium salt). The orange red product was collected, washed with 50 per cent saturated salt solution and dried over sulfuric acid. The overall yield was 11·8 g. (57 per cent). Purification was effected by precipitating the product from methanol with ether. Several purifications yielded a product that decomposed sharply at 146°. Repeated analysis showed the presence of 2 molecules of water which were not liberated below the decomposition temperature. Diels (1901) reports that fluorene-2-diazonium chloride also contains 2 molecules of water that are not liberated below decomposition temperature.

Found : Cl, 10·85, 11·01 ; Calc. for $C_{15}H_{16}ClN_3O_3$: Cl, 11·01 per cent.

For proof of structure, 2 g. of 2-acetylaminofluorene-7-diazonium chloride dihydrate were reflexed in 150 ml. of water for 30 minutes. The almost clear solution was filtered hot and a cream-white product precipitated from the cooled filtrate. Three recrystallizations from 50 per cent acetic acid resulted in a compound melting at 232°. Goulden and Kon (1945) reported that 2-hydroxy-7-acetylaminofluorene melts at 232°.

The dyes of this series were prepared by coupling the components in acid buffered, or basic aqueous solution, neutralization and filtration. The coupling in this series took somewhat longer and best results were obtained by allowing reactants to stand overnight. The yields ranged from 70 to 90 per cent. The products could be recrystallized from dilute Cellosolve, but highest purity was obtained from aniline, or benzyl alcohol. These compounds have not been previously reported. Constants and analyses are given in Table II.

TABLE IV.—*Results of Intraperitoneal Injection of Compounds III, IV and VI.*

Compound.	Time (hours).	Vol. of gastric juice (ml.).	pH of gastric juice.	Percentage found in stomach.	Color of serosa.	Color of gastric contents.
III	1·0	11·5	2	34	Orange	Yellow-orange.
	0·5	6·5	2	28	Yellow	,,
	0·25	10·7	2	30	Red	Red-orange.
IV	1·0	7·0	2	26	Colorless	,,
	0·5	4·9	2	24	Very slightly yellow	,,
VI	1·0	10·5	6–7	—	Yellow	Colorless.
	0·5	8·6	2	00	Red	Straw.
	0·25	9·4	2	00	Orange	,,
Propylene glycol	0·25	9·8	2	—	Colorless	,,

RESULTS AND DISCUSSION.

It will be seen from Table IV that Compound III appears in the gastric juice to the extent of 28 to 34 per cent of the amount injected. Secretion of the compound is substantially complete in 15 minutes. Apparently with the ligated pylorus an equilibrium is quickly established. Compound IV seems to be secreted to a slightly lesser extent, but this may not be significant.

The rather surprising result is the *non-secretion* of Compound VI. This was entirely unexpected, because it has a pK_B (8·5) which lies in the optimum secretion range (Ray and Jung, 1951). This indicates that the pK_B of maximum secretion in the fluorene series is much higher than it is in the benzene series. Because the serosa was colored, it was thought that this dye might have been reduced in the stomach at the azo link. An examination of the stomach contents for aminofluorene by the method of Westfall and Morris (1947) by Miss Mary F. Argus failed to show the presence of this substance, either free or conjugated.

While successful in leading us to potential carcinogens that are secreted in large amounts by the stomach, it is obvious that some factor (or factors) are involved other than the pK_B. It is true that the stomach of the rat may be less acidic than that of the dog, but this should only reduce the total concentration and not the relative order of absorption.

The use of a tricyclic molecule like fluorene in place of benzene leads to compounds with greatly reduced solubilities in dilute acid. The introduction of a third (insoluble) phase might well cause the stomach barrier to operate to exclude compounds of lower pK_B (greater basicity).

The demonstration that some of these compounds are secreted in rather large amounts in the stomach has encouraged us to set up long-time experiments for testing their carcinogenicity. The results will be reported on completion.

SUMMARY.

In the hope of obtaining a carcinogen that would be secreted by the stomach, 12 fluorene-azo dyes were prepared, of which the following are new compounds: fluorene-2-azo-2′,4′-diaminobenzene, fluorene-2-azo-2′-amino-4′-hydroxybenzene, 2-acetylaminofluorene-7-azo-2′,4′-diaminobenzene, 2-acetylaminofluorene-7-azo-5′ methyl-2′,4′-diaminobenzene, 2-acetylaminofluorene-7-azo-4′dimethylaminobenzene, 2-acetylaminofluorene-7-azo-2′,4′-dihydroxybenzene, and 2-acetylaminofluorene-7-azo-2′-amino-4′-hydroxybenzene.

Of 3 compounds injected intraperitoneally 2 were secreted by the stomach. Fluorene-2-azo-2′,4′-dihydroxybenzene and fluorene-2-azo-2′-methyl-4′-hydroxybenzene were present in gastric juice to the extent of 24 to 34 per cent. Neither fluorene-2-azo-5′-methyl-2′,4′-diaminobenzene nor its possible fission product, 2-aminofluorene (free or conjugated), was detected in the gastric juice.

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REFERENCES.

ADAMS, R., Editor-in-Chief.—(1944) 'Organic Reactions,' vol. ii. New York (John Wiley & Son , Inc.), p. 426.
ANASTAKRISHNAN, S. V., AND HUGHES, E. D.—(1935) *J. chem. Soc.*, 1607.
BIELENBERG, W., GOLDHAHN, H., AND PLUSKAL, H.—(1940) *Ber.*, **73**, 878.
BLATT, A. H., Editor.—(1943) ' Organic Syntheses,' *Coll.*, vol. ii. New York (John Wiley & Sons, Inc.), p. 447.
DAWSON, A. B., AND IVY, A. C.—(1925) *Amer. J. Physiol.*, **73**, 304
DIELS, O.—(1901) *Ber.*, **34**, 1758
FORSSMAN, J.—(1931) *Acta path. microbiol. scand.*, **8**, 16.
GOULDEN, F., AND KON, G. A. R.—(1945) *J. chem. Soc.*, 930.
KINOSITA, R., AND HARADA, M.—(1938) *Gann*, **32**, 225.
KORCZYNSKI, A., KARLOWSKI, G., AND KIERZIK, L.—(1927) *Bull. soc. chim.*, [4] **41**, 65.
MORGAN, G. T., AND THOMASON, R. W.—(1926) *J. chem. Soc.*, 2691.
MORRIS, H. P., AND DUBNIK, C. E.—(1950) *Cancer Res.*, **10**, 233.
Iidem AND JOHNSON, J. M.—(1950) *J. nat. Cancer Inst.*, **10**, 1201.
PACK, G. T., and LE FEVRE, R. G.—(1930) *J. Cancer Res.*, **14**, 167.
RAY, F. E., AND JUNG, MARY L.—(1951) *Brit. J. Cancer*, **5**, 358.
SCHULMAN, S.—(1949) *J. org. Chem.*, **14**, 382.
WESTFALL, B. B., AND MORRIS, H. P.—(1947) *J. nat. Cancer Inst.*, **8**, 17.
WILLIAMS, R. T.—(1947) 'Detoxication Mechanisms.' New York (John Wiley & Sons, Inc.), p. 151.

THE EFFECT OF AMINOPTERIN ON THE AUTOXIDATION OF DOPA.

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It has been shown that ethyl carbamate (urethane) and the water-soluble nitrogen mustard di-(2-chloroethyl)-methylamine hydrochloride have an accelerating effect on the autoxidation of 3, 4-dihydroxyphenylalanine (dopa) to melanin, and it has been suggested that the action of these substances in leukaemia may be due to a similar effect on other autoxidations, as opposed to enzymatic oxidations, occurring in leucopoiesis (Lea, 1950). It appeared that further evidence on this point might be obtained by investigating the action of 4-aminopteroylglutamic acid (aminopterin) on the oxidation of tyrosine and dopa.

METHOD.

The method used was the same as for the urethane and the nitrogen mustard. A flask containing the solution of the amino-acid and aminopterin was shaken in a Warburg bath at a temperature of 37·5° C. with a stroke of 6 cm. at a rate of 90 per minute, and melanin formation estimated at appropriate intervals by observing the extinction coefficients of the solution with an E.E.L. electro-colorimeter using a tri-colour green filter. Comparison was made with a solution of the amino-acid without aminopterin, and also with a solution of the amino-acid containing folic acid. All solutions were made in a buffer of pH 7·4 (colorimetric).

The effect of aminopterin on the oxidation of tyrosine by potato tyrosinase was first observed ; Table I gives the experimental layout.

TABLE I.—*Typical Experiments.*

	Flask No.				
	1.	2.	3.	4.	5.
Tyrosine %	0·005	0·005	0·005	0·005	0·005
Aminopterin %	0	0·0125	0·025	0	0
Folic acid %	0	0	0	0·0125	0·025
Buffer ml.	20	20	20	20	20
Enzyme ml.	1	1	1	1	1

(All percentages are weight/volume.)

After 5 hours no effect of either aminopterin or folic acid could be detected.

The experiment was repeated using a concentration of aminopterin and folic acid of 0·033 per cent. At this concentration both aminopterin and folic acid had an inhibiting effect on the tyrosine-tyrosinase reaction ; the results are shown in Table II.

TABLE II.—*The Oxidation of Tyrosine to Melanin in the Presence of* 0·033 *per cent of Aminopterin and Folic Acid.*

Increments of "E".

Time in minutes.	Tyrosine.	Tyrosine with aminopterin.	Tyrosine with folic acid.
60	·0165	·0148	·0119
120	·0386	·0301	·0265
180	·0514	·0433	·0391
240	·0646	·0569	·0468
300	·0755	·0653	·0574
360	·0838	·0710	·0654

It will be noted that the inhibition by folic acid is greater than that by aminopterin. These results were confirmed by two repetitions.

The effect of aminopterin and folic acid on the autoxidation of dopa was then investigated. (Dopa is the first intermediate product in the autoxidation of tyrosine to melanin. Tyrosinase is only necessary for the addition of the second hydroxyl to the benzene ring. After this has been done the oxidation will proceed without the aid of the enzyme (Raper, 1926).)

Concentrations of 0·0166 per cent of each of these substances were first used, the concentration of dopa being 0·005 per cent. Both substances produced an acceleration of the reaction, the effect of the aminopterin being much greater than that of folic acid. In succeeding experiments the concentration of aminopterin was reduced until a level of 0·00005 per cent was reached, at which level the accelerating effect was still evident (Table III).

TABLE III.—*The Effect of* 0·00005 *per cent Aminopterin on the Autoxidation of Dopa.*

Increments of "E".

Time in minutes.	Dopa.	Dopa plus aminopterin.
60	·0317	·0293
120	·0893	·0900
180	·1621	·1685
240	·2306	·2458
300	·2945	·3166

Finally a concentration of 0·0000125 per cent of aminopterin was used (approximately 1 part in 8,000,000), and at this level the accelerating effect was so small that it could not be distinguished from the probable experimental errors. This experiment was therefore made 17 times in all. On 15 occasions there was acceleration averaging approximately 0·3 per cent as compared with the control. On the assumption that the errors inevitably present will tend to operate equally in either direction, the probability of obtaining 15 results out of 17 in one direction is (approximately) only 1 in 500, so that the results are statistically significant.

The possibility of the acceleration being due to change in pH was reconsidered. The pH of the various solutions employed was estimated, electrometrically, using a glass electrode. The values found are given in Table IV.

TABLE IV.—*pH Values of Solutions.*

Solution.	pH.
Buffer	7·36
Dopa 0·005 %	7·28
Dopa 0·005% plus aminopterin 0·0125% .	7·26
Dopa 0·005% plus aminopterin 0·025% .	7·23
Dopa 0·005% plus folic acid 0·0125% .	7·25
Dopa 0·005% plus folic acid 0·025% .	7·23

There is a slight shift to the acid side of the original pH of the buffer solution. The autoxidation of dopa buffered at pH 7·2, 7·4 and 7·6 was therefore investigated. As anticipated, the reaction proceeded more slowly at the more acid pH (Table V).

TABLE V.—*The Effect of pH on the Autoxidation of Dopa.*

Time in minutes.	Increments of "E". pH 7·2.	pH 7·4.	pH 7·6.
60	·0134	·0179	·0271
120	·0465	·0586	·0871
180	·0876	·1091	·1631

It is evident that the accelerating effect of aminopterin and folic acid is not due to change in pH. What little change there is operates *against* such an acceleration.

DISCUSSION.

There is now evidence that three substances used in the treatment of leukaemia —urethane, nitrogen mustard and aminopterin—have an accelerating action on the autoxidation of dopa. Urethane and aminopterin have an inhibiting effect on the enzymatic oxidation of tyrosine, but only at concentrations much greater than those producing the acceleration of the autoxidation. The nitrogen mustard produced no inhibition of the enzymatic reaction even at a concentration of 0·2 per cent. Also folic acid produces this acceleration of the autoxidation. It might be supposed from this that any substances inhibiting the tyrosine-tyrosinase reaction would also accelerate the autoxidation of dopa. That this is not so is shown by the action of hydroquinone mono-benzyl ether, which inhibits both of these oxidations (Lea, 1951). These findings may have a bearing on the probable nature of the maturation factor in normal leucopoiesis.

Apparently the earliest work pointing to the presence of such a factor was that of Sabin, Cunningham, Doan and Kindwall in 1925. Since then it has been suggested that folic acid (Wilson, Doan, Saslaw and Schwab, 1942) and pyridoxine (Cantor and Scott, 1945) may be this factor. Dacie, Dresner, Mollin and White (1950) reported that after treatment of acute leukaemia by aminopterin there appeared to be a return to normal haemopoiesis. It is now suggested that this factor, whatever its nature, acts by promoting autoxidative processes in the normal production of leucocytes. Such a suggestion allows the inclusion of folic acid with substances recognized as therapeutic agents in the leukaemias. It is to be noted that folic acid ceased to produce an accelerating effect on the

autoxidation of dopa at a concentration of approximately 1 part in 100,000, whereas the effect of aminopterin was still obvious at a level of 1 part in 2,000,000, and detectable by statistical methods at a level of 1 part in 8,000,000.

It appeared that still further evidence bearing on this suggestion might be obtained by comparing the degrees of acceleration of the autoxidation of dopa produced by urethane, nitrogen mustard and aminopterin. This was done, and it was found that the accelerating effects of these substances were in the ratio of urethane 1, nitrogen mustard 100, aminopterin 1000. These figures are approximate, and indicate only the order of the differences. It is of interest to compare these orders of magnitude of acceleration with the amounts of these substances used therapeutically, and with their clinical effects.

It must be emphasized that there is no suggestion that the autoxidation of dopa plays any part in either normal or abnormal leucopoiesis ; or that any one of the three substances so far investigated is the actual maturation factor. All that it is intended to convey here is that the maturation factor may be concerned with some form of autoxidative process, and that leukaemia may be a result of its dysfunction. These suggestions are put forward only as a hypothesis for investigation, with the further suggestion that it might be of interest, and possibly of advantage, to investigate the effects on the reticuloses of other substances which accelerate autoxidations. There appears to be no association between these findings and the release factor of normal, or abnormal, leucopoiesis.

SUMMARY.

(1) Aminopterin and folic acid inhibit the tyrosine-tyrosinase reaction, the effect of folic acid being greater than that of aminopterin.

(2) Aminopterin and folic acid accelerate the autoxidation of dopa, the effect of aminopterin being much greater than that of folic acid.

(3) It is suggested that these results, taken in conjunction with similar findings for urethane and di-(2-chloroethyl)-methylamine hydrochloride, admit of the hypothesis that the maturation factor in leucopoiesis is concerned with the promotion of autoxidations.

(4) It is suggested that the study of other substances having this accelerating effect on autoxidations might help in the solution of the problem of the leukaemias.

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REFERENCES.

CANTOR, M. M., AND SCOTT, J. W.—(1945) *Canad. med. Ass. J.*, **52**, 368.

DACIE, J. V., DRESNER, E., MOLLIN, D. L., AND WHITE, J. C.—(1950) *Brit. med. J.*, i, 1447.

LEA, A. J.—(1950) *Brit. J. Cancer*, **4**, 341.—(1951) *Nature*, **167**, 906.

RAPER, H. S.—(1926) *Biochem. J.*, **20**, 735.

SABIN, F. R., CUNNINGHAM, R. S., DOAN, C. A., AND KINDWALL, J. A.—(1925) *Johns Hopk. Hosp. Bull.*, **37**, 14.

WILSON, H. E., DOAN, C. A., SASLAW, S., AND SCHWAB, J. L.—(1942) *Proc. Soc. exp. Biol., N.Y.*, **50**, 341.